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In vitro assembly of infectious nucleocapsids of bacteriophage ϕ6: Formation of a recombinant double-stranded RNA virus

(packaging/replication/genomic segments)

VESA M. OLKKONEN*, PAUL GOTTLIEB†, JEFFREY STRASSMAN†, XUEYING QIAO†, DENNIS H. BAMFORD*, AND LEONARD MINDICH†‡

*Department of Genetics, University of Helsinki, Arkadiankatu 7, SF-00100, Helsinki, Finland; and †Department of Microbiology, Public Health Research Institute, 455 First Avenue, New York, NY 10016

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ABSTRACT **A system is described for assembling infectious bacteriophage ϕ6 nucleocapsids *in vitro*. Procapsids encoded by cDNA copies of genomic segment L in *Escherichia coli* were used to package and replicate viral RNA segments. The resulting filled particles were shown to be capable of infecting host cell spheroplasts after incubation with purified nucleocapsid shell protein P8. The infected spheroplasts yielded infectious virions. A modified cDNA-derived RNA segment was inserted into virions by this method. The resulting infectious virions contained the same 4-base-pair deletion as the modified cDNA. These findings support the contention that the preformed procapsids are the "machine" that replicates the ϕ6 genome, by showing that the cDNA-derived procapsids are competent to package and replicate RNA properly.**

Bacteriophage ϕ6 is a double-stranded RNA (dsRNA) virus of *Pseudomonas phaseolicola* (1). Its segmented genome, consisting of three separate pieces (2, 3), is associated with a complex isometric nucleocapsid (NC) structure (4, 5). The NC has five protein components, one of which (protein P8) forms the outer shell of the particle (4, 5). The other four proteins (P1, P2, P4, and P7) comprise an inner particle that displays the viral transcriptase and replicase activities (6–10). The ϕ6 NC is enclosed in a lipid–protein envelope, the components of which have vital functions in the initial steps of the phage infection (1, 11–13).

The internal NC proteins, P1, P2, P4, and P7, are encoded by the large (L) genomic segment (14, 15) and are synthesized early in ϕ6 infection (5). Particles (procapsids) consisting of these four proteins and displaying RNA polymerase activity can be purified from *Escherichia coli* or *P. phaseolicola* cells harboring cDNA copies of the L segment in an expression vector (9, 10). Protein P1 forms the dodecahedral framework of the procapsids, to which the other proteins are attached (9, 17, 18). Defective particles lacking protein P2 have no polymerase activity, suggesting that this protein forms the active site of the enzyme. The cDNA-derived procapsids are capable of packaging and replicating viral (+)-strand RNA to double-stranded genomic segments, as well as of producing transcripts using the dsRNA formed as template (10).

Recently, a technique for infecting *P. phaseolicola* spheroplasts with the purified ϕ6 NC has been developed (19). Furthermore, the infectivity of the NC is dependent on the presence of the shell consisting of protein P8 (V.M.O., P. M. Ojala, and D.H.B., unpublished work). In that study, a method was devised for restoring the infectivity of noninfectious *in vitro* uncoated NCs (NC cores devoid of P8) by assembly of the purified coat protein onto the particles. In this investigation, we combine the above techniques of *in vitro* dsRNA replication, coat protein assembly, and NC infection. We show that the cDNA-derived ϕ6 polymerase complexes that have replicated the viral RNA *in vitro* can be turned infectious by coat protein assembly. This is confirmed through the use of a modified RNA segment with a 4-base-pair (bp) marker deletion, produced from cDNA of the medium (M) segment with T7 RNA polymerase. The results that show that cDNA-derived procapsids are capable of replicating the viral RNA, packaging it properly, and yielding particles competent for coat protein assembly. Furthermore, the procapsids are biologically active, being capable of initiating a productive infection.

MATERIALS AND METHODS

Bacterial Strains, Phage, and Plasmids. *P. phaseolicola* strain HB10Y (1) was used as the host for ϕ6 lysate preparations and for phage titration and for the determination of infectious centers in the NC infection experiments. A receptorless ϕ6-resistant derivative of HB10Y, HB MP0.16 (20), was used for the preparation of spheroplasts that can be infected by ϕ6 NC.

Bacteriophage ϕ6 (1) was used for the preparation of phage NCs used in single-stranded RNA (ssRNA) production and as the source of the NC coat protein, P8.

The expression plasmid pLM450 (10) harboring ϕ6 L-segment cDNA was used for the production of phage procapsids in *E. coli* JM109. Strain JM109 was the host for the propagation of recombinant plasmids. *E. coli* strain CJ236 is a *dut*, *ung* derivative that is used for the generation of uracil-containing DNA in phagemids (21).

Preparation of ϕ6 ssRNA. ϕ6 ssRNA was synthesized by an *in vitro* transcription reaction essentially according to Emori *et al.* (22), using NCs prepared as described by Mindich *et al.* (23). The resulting mixture of ssRNA and dsRNA was fractionated on a cellulose column with elution buffers containing decreasing concentrations of ethanol, according to Franklin (24). The ssRNA eluted at 20% ethanol was concentrated by ethanol precipitation and dissolved in water. The individual ssRNA species were isolated from the original transcription mixture by rate zonal sucrose gradient centrifugation [5–20% (wt/vol) sucrose in 50 mM Tris Cl, pH 7.5/300 mM NaCl; Beckman SW41 rotor, 17 hr, 4°C, 21,000 rpm). The gradients were fractionated and the ssRNA zones were identified in composite gels (25). The peaks were collected, concentrated by ethanol precipitation, and dissolved in water. The concentration of the unfractionated ssRNA preparation used was 50–75 μg/ml; the values for the individual fractionated ssRNAs were somewhat lower (see Fig. 1).

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Abbreviations: NC, nucleocapsid; dsRNA, double-stranded RNA; ssRNA, single-stranded RNA.

‡To whom reprint requests should be addressed.

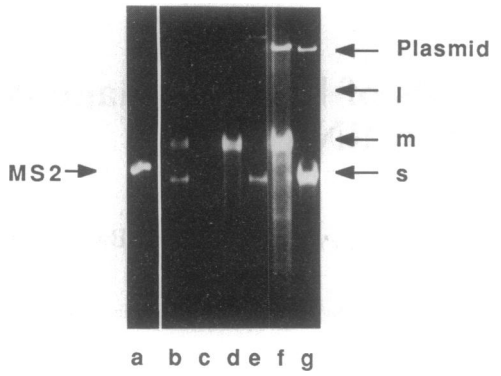


FIG. 1. The ϕ6 ssRNA preparations used for *in vitro* packaging and replication. mRNA was produced by an *in vitro* reaction with purified NCs, and the resulting mixture of ssRNA and dsRNA was fractionated on a cellulose column. The individual ssRNA species were isolated by rate zonal centrifugation in sucrose gradients. The RNAs were analyzed by electrophoresis in composite polyacrylamide/agarose gels. Lanes: a, MS2 RNA standard; b, ϕ6 ssRNA; c, the *l* species; d, the *m* species; e, the *s* species; f, the synthetic *m* ssRNA with a 4-bp deletion produced from M-segment cDNA in plasmid pLM674 with T7 RNA polymerase; g, a synthetic *s* ssRNA. The mobilities of the ϕ6 mRNAs as well as that of the *Xba* I-cut plasmid pLM674 used for M cDNA run-off transcription (lane f), are indicated at right. The *s* ssRNA species in lane e is contaminated with a small amount of the L dsRNA.

Construction of Plasmid pLM674. The construction of a plasmid that would yield a transcript identical to the viral *m* ssRNA species is described in Fig. 2. The original cDNA material was cloned into the pBR322 *Pst* I site with poly(dG) tails (23). The sequences of fragments 82 and 316 contained the complete 5′ and 3′ ends of segment M, respectively (16). Fragment 600 was prepared by ligating fragments 82 and 67. Oligonucleotides used for both the polymerase chain reaction (PCR; ref. 26) and directed mutagenesis reactions (21) were prepared at the Public Health Research Institute, New York, by Y. K. Yip. The final construction is in plasmid pT7T319U (Pharmacia). This plasmid has promoters for both T7 and T3 polymerases as well as the *lacZ′* element of pUC19 (27). It also has the replication origin of phage f1. All sequence changes were verified by sequence determination.

Preparation of Procapsids. ϕ6 procapsids were prepared according to Gottlieb *et al.* (9), using the expression plasmid pLM450 (10) propagated in *E. coli* JM109. The procapsid preparation used contained protein at 400 μg/ml as determined by the Bradford assay (28).

Purification of Protein P8. The NC coat protein P8 was purified by stepwise disruption of ϕ6 followed by immunoaffinity chromatography (V.M.O., P. M. Ojala, and D.H.B., unpublished work), as follows. NCs were disrupted by treatment with 25 mM EDTA and the soluble protein fraction was isolated from sucrose gradients (29). The soluble proteins were passed through an immunoaffinity column with the P8-specific monoclonal antibody 8Q2 linked to cyanogen bromide-activated Sepharose CL-4B (Pharmacia), and the bound P8 was eluted with 100 mM glycine/HCl, pH 3.0. The eluted protein was dialyzed against 20 mM potassium phosphate, pH 7.4/0.5 mM EDTA. The protein was concentrated with polyethylene glycol (PEG) 20,000 and stored at −70°C. The preparation used contained protein at 200 μg/ml and showed no contaminating bands in stained gels.

Polymerase Reaction Conditions. The ϕ6 RNA polymerase reaction mixtures contained 50 mM Tris Cl, pH 8.2; 2 mM $MnCl_2$ or 3 mM $MgCl_2$; 100 mM NH_4OAc; 20 mM NaCl; 5 mM KCl; 5 mM dithiothreitol; 0.1 mM Na_2EDTA; 1 mM each ATP, GTP, CTP, and UTP; 5% PEG 4000, and 40 mg of Macaloid per ml (30). To each 25-μl reaction mixture, 500–750 ng of ϕ6 ssRNA and 1.4 μg of procapsid protein were added. In the experiment where the cDNA-derived M-segment RNA was used, the amounts of natural *l* and *s* ssRNAs added were about one-third of the amount in the case of the natural ssRNA mixture; the amount of the synthetic *m* transcript was 5–10 μg. The reaction mixtures were routinely incubated at 28°C for 75 min.

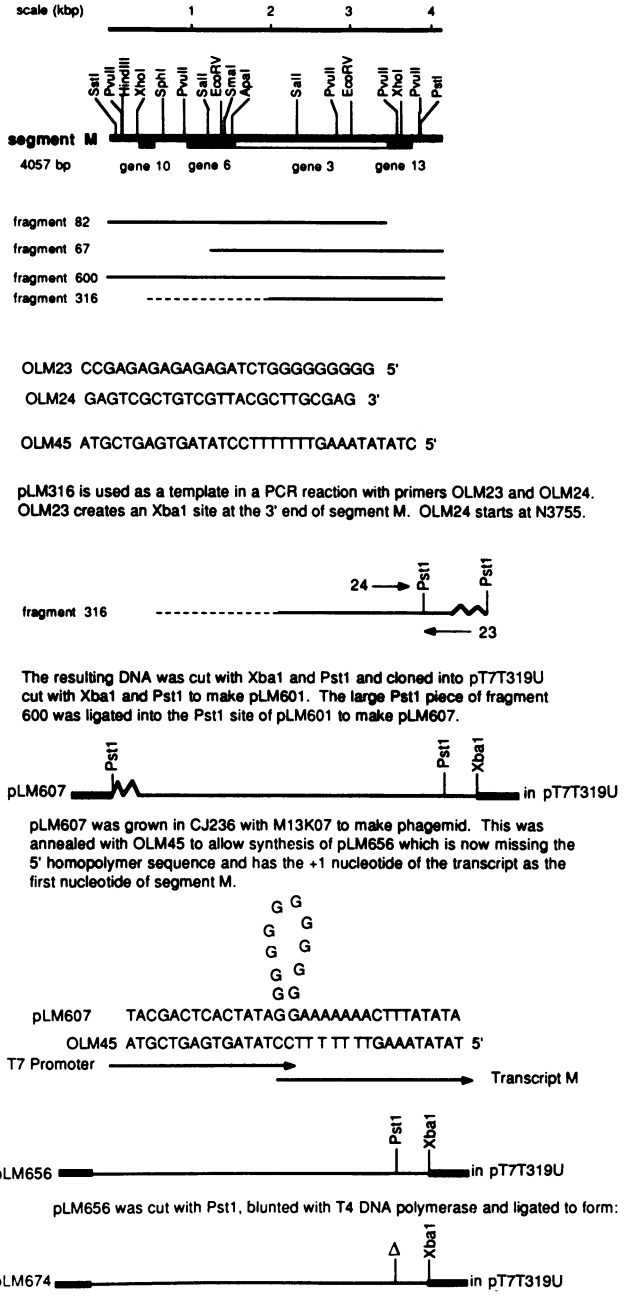


FIG. 2. Construction of plasmid pLM674. After cutting with *Xba* I and blunting with mung bean nuclease, plasmid pLM656 produces a transcript that is identical to the natural transcript of segment M. Plasmid pLM674 is missing 4 bp from the *Pst* I site. When transcribed in the same manner as pLM656 it produces a transcript that is identical with the natural transcript of segment M except for the 4-base deletion at the *Pst* I site. PCR, polymerase chain reaction; N3755, nucleotide 3755.

Assembly of Coat Protein. Protein P8 was assembled on the packaged procapsids in 31.5-μl mixtures containing 10 μl of the specific polymerase reaction mixtures and a standard amount of 2.2 μg of purified P8. In the optimization experiments, various concentrations of KCl, NaCl, NH_4Cl, and $CaCl_2$ were added to the assembly mixture. The amount of

P8, the time of coat protein assembly, and the temperature were varied. The final composition of the assembly mixture chosen (including the components carried over from the polymerase reaction and from the P8 preparation) was 15.9 mM Tris Cl, pH 8.2/0.95 mM $MgCl_2$/0.21 mM Na_2EDTA/6.3 mM NaCl/97 mM KCl/95 mM NH_4Cl/1.6 mM dithiothreitol/7.0 mM potassium phosphate, pH 7.4/31.7 mM NH_4OAc/0.32 mM each NTP/1.6% PEG 4000/0.71 mM $CaCl_2$. The reaction was routinely allowed to proceed for 1 hr at 24°C.

NC Infection. HB MP0.16 cells were rendered competent for ϕ6 NC infection as described (19). To each 120-μl aliquot of cells, 20 μl of one of the coat protein assembly mixtures was added, and the infection was allowed to proceed in 35-μl droplets on Millipore VSWP02500 filters on LB plates overlaid with LB top agar/3% lactose/20 mM potassium phosphate, pH 7.2, at room temperature for 60 min. Thereafter, the cells were washed once with 1 ml of LKSB buffer (19) and resuspended in 100 μl of the same buffer, and infectious center titers were determined on HB10Y "lawns." To confirm that we were really measuring infectious centers and not plaque-forming units, control infection mixtures were treated for 15 min with the phage-neutralizing monoclonal antibody 303 (31), before the washing and plating steps. In each infection experiment, ϕ6 NCs (V.M.O., P. M. Ojala, and D.H.B., unpublished work) were used to control the competence of the cell preparations for infection. The control infection mixtures consisted of 60 μl of cells and 0.3 μg of added NC protein.

RNA Sequence Analysis. ssRNA for sequencing was produced using the *in vitro* NC transcriptase reaction as described above. Sequence analysis was performed by the dideoxy chain-termination method (32) modified as described by Hamlyn *et al.* (33) but with ITP instead of GTP (15). The synthetic oligodeoxynucleotide used as primer was 5'-CGCCTCAAGCAACTGCATTG-3', annealing with a sequence starting 50 bp downstream from the 4-bp *Pst* I-site deletion in the synthetic *m* ssRNA (see Fig. 2).

RESULTS

Assembly Conditions for the Shell Protein P8. The optimal conditions for the assembly of protein P8 onto the cDNA-derived procapsids containing *in vitro* replicated dsRNA were determined by measuring the number of infectious centers formed from incubation of these assembly mixtures with cells competent for NC infection. In the optimization experiments, a mixture of ϕ6 ssRNAs produced by *in vitro* transcription with the NCs was used as the replication template. After the replication-packaging reaction, purified protein P8 was added in the presence of Ca^{2+} ions. Finally, *P. phaseolicola* spheroplasts were incubated with the coat protein assembly mixture, and the infectious centers formed were determined on host cell lawns.

Optimization of the coat protein assembly included experiments where the amount of P8 used, the ionic composition of the reaction mixture, and the time allowed for the reaction were varied. The presence of Ca^{2+} ions was required for generation of infectivity (Table 1), the optimal concentration being around 0.7 mM. Very few infectious centers were formed in the absence of Ca^{2+}. Addition of KCl or NH_4Cl greatly increased the number of infectious centers formed; the optimal concentration was 150–300 mM. NaCl did not promote the infectivity. The optimal coat protein assembly mixture (31.5 μl) consisted of 10 μl of polymerase reaction mixture and 2.2 μg of P8. The additional components were 95 mM KCl, 95 mM NH_4Cl, and 0.71 mM $CaCl_2$. The highest levels of infectivity were reached with final P8 concentrations >35 μg/ml (1.1 μg added per 31.5 μl). The shortest assembly time tested, 5 min, already gave the maximal level of infectivity; the standard time used was 60 min at 24°C. The assembly reactions were carried out at 15, 24, and 28°C with no significant differences in yield of infectious particles.

The frequency of infectious centers obtained with the *in vitro* assembled particles at the optimal conditions was about 1.4×10^4 per ml. The virus-derived NC preparations with the same particle concentration gave a frequency of 1.5×10^7 per ml.

RNA Polymerase Reaction. Generation of infectivity in the system was found to be totally dependent on the presence of template ssRNA and of procapsids added in the polymerase reaction. The procapsids with replicated dsRNA were absolutely noninfectious without the coat protein (Table 1). The infectivity showed a time dependency in relation to the replication reaction, and the response to the concentration of ssRNA used in the reaction was exponential. Two types of polymerase reactions were performed: in the beginning of the work the mixture of Gottlieb *et al.* (10), containing $MnCl_2$, was used. Soon, however, we noticed that a reaction mixture with $MgCl_2$ gave much higher numbers of infectious centers (see Table 1).

Introduction of a Synthetic dsRNA Segment into Infectious Virus. A plasmid was constructed that contained the cDNA copy of ϕ6 genomic segment M under the control of a T7 promoter (Fig. 2). The plasmid was cut with *Pst* I and the staggered ends were blunted with T7 DNA polymerase. The ends were then self-ligated, resulting in a 4-bp deletion in the M-segment 3' noncoding region. The plasmid was cut with *Xba* I and the 5' overhang was removed with mung bean nuclease. Transcripts were produced with T7 RNA polymerase (Fig. 2). This ssRNA was combined with natural *l* and *s* ssRNAs purified in sucrose gradients (Fig. 1). The mixture was used in an *in vitro* replication–packaging reaction. Coat protein P8 was then assembled onto this product for an infection experiment. The control reaction with only the natural *l* and *s* ssRNAs gave no infectious centers, whereas 17 were found in the reaction mixture containing the added synthetic *m*-segment RNA. Purified phage stocks were prepared from five of these recombinant virus plaques. Nucleocapsid preparations of these stocks were used to generate ssRNA transcripts, and the base sequence in the region of the *Pst* I site was determined. The RNA sequences of all five virus preparations displayed the 4-bp deletion introduced as a marker in the M-segment cDNA (Fig. 3). Wild-type phage showed the expected complete *Pst* I site, confirming that the recombinant RNA segment had become part of the genome of infectious virions.

DISCUSSION

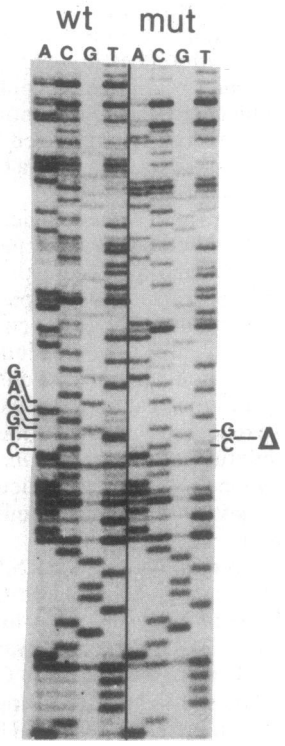
ϕ6 RNA polymerase complexes (procapsids) that are formed in *E. coli* exhibit *in vitro* dsRNA replicase and transcriptase

Table 1. Transfection of spheroplasts with recombinant procapsids

RNA polymerase reaction	P8 assembly	Infectious centers per ml
With Mg^{2+}, no ssRNA	Optimal	0
With Mg^{2+}, no procapsid	Optimal	0
With Mn^{2+}	Optimal	7.3×10^2
With Mg^{2+}	No P8	0
With Mg^{2+}	No Ca^{2+}	7
With Mg^{2+}	Optimal	1.4×10^4
With Mg^{2+}	Optimal + Mn^{2+}	1.4×10^4
Control NC preparation		1.5×10^7

The RNA polymerase reactions were performed under the conditions described in *Materials and Methods*, with the exception that the ssRNA or the procapsids were omitted from control samples. Thereafter, the polymerase reaction mixtures were incubated with protein P8 under the optimal conditions given in *Materials and Methods*.

A



B

Wild type CTATACAACXACTGCAGCTGCTCATACGA

Mutant CTATACAACXAC GCTGCTCATACGA

FIG. 3. Sequence analysis of a recombinant virus. Nucleocapsids purified from wild-type and recombinant phage isolates were used for ssRNA production by *in vitro* transcription. The ssRNAs were used for sequence analysis. (*A*) The four lanes on the left show the sequence of a wild-type (wt) ϕ6 isolate: the lanes on the right show the sequence of a recombinant mutant (mut) virus. The *Pst* I site used for making the marker deletion in M-segment cDNA is indicated at left. The deletion is indicated (Δ) at right. (*B*) The nucleotide sequences in the region of the *Pst* I site under investigation, read from the gel in *A*. The *Pst* I site is indicated by a line above the wild-type sequence: the 4-bp deletion introduced in the M-segment cDNA (see Fig. 2) is seen in the recombinant virus sequence.

activity (9, 10). The procapsid binds and packages viral message from solution and performs (−)-strand synthesis upon these templates. The resulting dsRNA remains within the procapsids, which then start to function as transcriptase, synthesizing additional message by strand displacement (34, 35) using the dsRNA as template.

In this investigation, we have demonstrated that it is possible to generate infectious nucleocapsids by incubating P8 with packaged procapsids. This confirms that the cDNA-derived ϕ6 procapsids are capable of packaging the three RNA segments properly, replicating the viral RNA, and yielding particles competent to receive coat protein. The particles are infectious in the NC infection system developed by Ojala *et al.* (19) showing that the cDNA-derived procapsids are biologically active *in vivo*—i.e., are capable of transcription in the infected spheroplasts, leading to productive infection. The results were confirmed by introducing a cDNA-derived M-segment RNA marked by a 4-bp deletion into infectious virions. The presence of the synthetic segment in the virus was verified by RNA sequence analysis.

Our results support a model in which the four procapsid proteins are sufficient for the packaging, replication, and transcription of the ϕ6 genomic segments. This model is remarkably similar to that proposed many years ago by Acs *et al.* (36) for the replication of the reovirus genome.

Procapsids that replicated and packaged RNA in the presence of Mg^{2+} produced a higher number of infectious centers than those incubated in a Mn^{2+} replication mixture. Recent results by Gottlieb *et al.* (10) show that the Mn^{2+} reaction displays less stringency. We suspect that the majority of the particles produced in the Mn^{2+} reaction are defective, or alternatively, the conformation of the particles does not allow efficient assembly of the coat protein.

The frequency of infectious centers formed from the virus-derived control NCs was about 1000 times greater than that from the *in vitro* assembled particles on the basis of the relative amounts of procapsid proteins. This is due to the fact that only a minor proportion of the procapsids have replicated and packaged RNA in the *in vitro* reaction and that not all of the packaged particles have all three segments (10).

A system has been described by Luytjes *et al.* (37) and Enami *et al.* (38) in which a cDNA-derived influenza A (−)-strand RNA segment complexed with purified polymerase and nucleoprotein components is transfected into cells infected by a helper virus. The cDNA-derived segment was shown to be recovered in the virus progeny. A significant difference between our system and that described above is that in the ϕ6 system the entire RNA replication process takes place *in vitro*. No helper virus is thus needed, since the transfecting agent (NC) in this case contains all the genomic segments in a functional form. If one of the segments is modified, it will be present in the entire virus progeny. Therefore any mutant RNA, assuming that its replication and transcription recognition sequences are intact, can with this technique be transferred to infectious virions. The missing vital phage functions can be provided from an expression plasmid in the host strain.

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