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Isolation and Characterization of the Smallest Bacteriophage P4 Derivatives Packaged into P4-Size Head in Bacteriophage P2-P4 System

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Bacteriophage P4, a satellite phage of coliphage P2, is a very useful experimental tool for the study of viral capsid assembly and *cos*-cleavage. For an *in vitro cos*-cleavage reaction study of the P2-P4 system, new shortened and selectable markers containing P4 derivative plasmids were designed as a substrate molecules. They were constructed by swapping the non-essential segment of P4 DNA for either the kanamycin resistance (*kmr*) gene or the ampicillin resistance (*apr*) gene. The size of the genomes of the resulting markers were 82% (P4 *ash8 del*RI:: *kmr*) and 79% (P4 *ash8 del*RI:: *apr*) of the wild type P4 genome. To determine the lower limit of genome size that could be packaged into the small P4-size head, these shortened P4 plasmids were converted to phage particles with infection of the helper phage P2. The conversion of plasmid P4 derivatives to bacteriophage particles was verified by the heat stability test and the burst size determination experiment. CsCl buoyant equilibrium density gradient experiments confirmed not only the genome size of the viable phage form of shortened P4 derivatives, but also their packaging into the small P4-size head. P4 *ash8 del*RI:: *apr* turned out to be the smallest P4 genome that can be packaged into P4-sized head.

Keywords: bacteriophage P2-P4, *Escherichia coli*, *cos*-cleavage, packaging, P4-size head

The satellite bacteriophage P4 requires a helper bacteriophage such as bacteriophage P2 for its lytic growth (Guttman *et al.*, 2004). It depends on all the known morphogenetic gene products of bacteriophage P2 for the production of its capsid and tail (Lindqvist *et al.*, 1993). Consequently, these two bacteriophages appear to be the same particle under electron microscope except for their head size. The large P2-size head is 60 nm in diameter and the small P4-size head is 45 nm in diameter (Dokland *et al.*, 1992; Wang *et al.*, 2006). The volume of the P4-size head is about one-third of that of the P2-size head. Both heads are made of the same major capsid protein encoded by *N* gene of P2 (Six *et al.*, 1991). Bacteriophage P4's *sid* gene is responsible for the small P4-size head assembly with the gene product of N (g.p.N) of bacteriophage P2 (Nilssen *et al.*, 1996). Genetic and molecular biological studies have revealed that the Sid proteins act as outer-scaffolding proteins in small P4-size head assembly (Marvik *et al.*, 1995).

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Similar to other icosahedral phages, the heads of P2 and P4 particles are tightly packed with phage genomic DNA. As the volume of the P2-size head is three times greater than that of the P4-size head, a 33.6 kb long P2 genome is packaged into the large P2-size head whereas a 11.6 kb long P4 genome is packaged into the small P4-size head (Bertani and Six, 1988). The lower limit on the size of P2 DNA compatible with P2-size head has been established with the isolation of some deletion mutants of P2. One of these deletion mutants has lost 14% of its DNA, but is still viable (Bertani and Chattoraj, 1980).

In the case of the P4-size head, the study of P4 deletion mutants delimited the lower range of DNA size to be packaged into the small head. P4 *del22*, one of deletion mutants of P4, is known to be the smallest P4 derivative to be packaged into the small head so far (Raimondi *et al.*, 1985). The deletion removes about 1.7 kb of DNA from the nonessential region of the P4 genome. The deleted portion of DNA in P4 *del22* is approximately 14.6% of the P4 genome.

For the preparation of substrate for *in vitro cos*-cleavage reaction of the bacteriophage P2-P4 system, we designed plasmid P4 derivatives which have

antibiotic markers to facilitate selection. During the substrate preparation, some plasmid P4 derivatives with genome sizes shorter than P2 *del22* were constructed. We concentrated on these derivatives in order to find the lower limit of P4 genome packaging into the small P4-size head.

In this paper, we report the construction and isolation of those shortened plasmid P4 derivatives, which are 79% and 82% of P4 genome size. We converted them into biologically active bacteriophage P4 packaged into the P4-size head and found that they were the smallest P4 derivatives that could be packaged into the small P4-size head.

Materials and Methods

Bacterial strains, plasmids and bacteriophages

Escherichia coli strain C1a was used as the host cell for plasmid type propagation of P4 (Sasaki and Bertani, 1965). *E. coli* C295 and C353, which harbor the P2 prophage, were used as the host cells for the propagation of bacteriophage P4 (Kim *et al.*, 2001).

Bacteriophage P4 *ash8* was the starting material for the construction of new P4 derivatives (Lin, 1984). As the *ash8* mutation enables P4 to establish itself as a multi-copy plasmid, plasmid P4 *ash8* harboring *E. coli* C375 were cultivated for plasmid P4 isolation. Bacteriophage P2 *vir1* was used for the induction of bacteriophage type P4 derivatives (Bertani and Six, 1988).

Plasmids pUC4-K (Norrander *et al.*, 1983) and Litmus38 (NEB, USA) were the sources for the kanamycin resistance (*kmr*) gene cassette and the ampicillin resistance (*apr*) gene respectively.

Media

Culture media were Luria Bertani (LB) broth or LB agar. LB supplemented with anti-P2 serum (neutralization constant k = 1/min) (LBSe) was used in one-step growth experiments. When antibiotic selection was needed, *E. coli* bacteria were grown in LB broth or on LB agar supplemented with 50 μg/ml ampicillin or kanamycin. The incubation temperature was 37°C, unless otherwise noted.

DNA manipulations

Restriction enzymes, S1 nuclease and T4 DNA ligase were used according to the manufacturer's specifications.

Plasmid P4 *ash8* DNA from *E. coli* C375 was isolated by the alkaline lysis method. Other recombinant DNA techniques were carried out according to Sambrook *et al.* (2001).

Construction of P4 ash8 delRI:: kmr

Plasmid P4 *ash8* DNA was digested with *Eco*RI to produce an 8.2 kb fragment containing all of the essential genes of P4. In order to obtain P4 *ash8 del*RI:: *kmr*, the 8.2 kb *Eco*RI digested P4 *ash8* DNA was ligated with the 1.24 kb kanamycin resistance (*kmr*) gene cassette from plasmid pUC4-K by *Eco*RI cleavage. Kanamycin resistant colonies were selected in DNA transformation with *E. coli* C1a competent cells. Restriction enzyme digestion analysis of transformants confirmed the construction of P4 *ash8 del*RI:: *kmr*. The calculated size of the resulting P4 *ash8 del*RI:: *kmr* was 9.5 kb.

Construction of P4 ash8 delRI:: apr

Plasmid P4 *ash8* DNA was digested with *Eco*RI to produce an 8.2 kb fragment. This DNA segment was treated with S1 nuclease to make both ends blunt. To obtain P4 *ash8 del*RI:: *apr*, we ligated the 8.2 kb blunt-ended P4 *ash8* DNA fragment with the 973 bp ampicillin resistance (*apr*) gene containing the DNA fragment from plasmid Litmus38 by *Ssp*I cleavage. Ampicillin resistant colonies were selected in DNA transformation with *E. coli* C1a competent cells. Restriction enzyme digestion analysis of transformants confirmed the construction of P4 *ash8 del*RI:: *apr*. The calculated size of the resulting P4 *ash8 del*RI:: *apr* was 9.18 kb.

Preparation of bacteriophage stock of P4 derivatives

Bacterial lawns of *E. coli* C1a harboring P4 *ash8 del*RI:: *kmr* or P4 *ash8 del*RI:: *apr* were spotted with one drop of helper bacteriophage, P2 *vir1*, and incubated at 37°C overnight. The next morning, an area of lysis appeared around the spot. Agar plugs were taken from the lysis area and transferred to 1 ml LB. Two drops of chloroform were added to kill remaining bacteria. This phage suspension was plated using *E. coli* C353 as a P4 indicator to obtain a single plaque.

To prepare the bacteriophage P4 derivative stock, *E. coli* C295 cells were grown in 25 ml LB supplemented with $CaCl_2$ (final concentration 1.4 mM) and $MgSO_4$ (final concentration 2.0 mM) to about 1×10^8 CFU (colony forming units)/ml at 37°C and inoculated with a single cored plaque. The optical density (OD) at 600 nm of the culture was monitored using a spectrophotometer (Spectronic 20, Bousch and Lomb, USA). At the onset of lysis (beginning of OD drop), EGTA was added to a final concentration of 0.01 M. At the completion of lysis, unlysed cells and debris were removed by low speed centrifugation (10 min at 5,000 rpm using SS-34 rotor) and the supernatant was centrifuged for 2 h in a LE-80K ultracentrifuge (Beckman, USA) at 73,000 × g (type 70Ti rotor) to obtain a phage pellet. The bacteriophage stock of the P4 derivative was prepared by resuspending the pellet in 1 ml of 75 mM $MgCl_2$ (Kim, 1998).

Transduction experiments

An *E. coli* C1a lawn was prepared on L agar containing kanamycin or ampicillin. One drop of P4 derivative phage stock was put on the lawn. The plate was incubated at 37°C overnight. The following morning, P4 derivative phage infected *E. coli* C1a cells had formed colonies on the spot area without any helper phage P2. These colonies were the transductants harboring antibiotic resistant P4 derivative plasmid.

Heat stability test for P4 derivatives

Approximately 10^{10} phages of P4 derivative stocks were diluted 1:100 in SCC buffer (0.015 M citrate and 0.15 M NaCl, pH 7.0) and were titrated for P4 PFU (plaque forming units) using C353 as an indicator. Diluted phage samples were incubated at 45°C for 1 h. After heat treatment, the titers of phage samples were determined. Stability was calculated as the ratio of viable phage titer before and after heat treatment.

One-step growth experiments

One-step growth experiments were done according to Six and Klug (1973). Host cells were grown in LB to about 6×10^7 CFU/ml, concentrated to about 2×10^8 CFU/ml in LB supplemented with $CaCl_2$ (final concentration, 5 mM) and assayed for colony former. Infection was started (t=0 min) with the addition of phages, at an MOI (multiplicity of infection) between 3 and 8, to 0.6 ml cell culture. The culture was incubated at 37°C (unless otherwise stated). At t=7 min, 0.1 ml culture was diluted 10 fold into LBSe to neutralize most of the unadsorbed phages. The remaining culture was diluted about 15 fold by adding 7 ml LB and returned to 37°C. The OD at 600 nm of wavelength of this culture was monitored at 10 min intervals to determine onset and time course of cell lysis. At t=14 min, the LBSe culture was diluted 10,000 fold by two successive 100 fold dilutions with LB. A portion of the last dilution tube (usually 2 ml) was saved for the yielder frequency plaque assays. The last dilution tube was incubated at 37°C until the OD monitoring indicated completion of cell lysis, then phage production was assayed from this tube (directly or after further 10, 40, or 100 fold dilution as required). Streptomycin was added to kill any remaining host cells in the phage production assay.

The yielder frequency is the number of phage yielding cells expressed as the percentage of infected cells. The latter is estimated from the MOI. In the case of low MOI, the Poisson distribution was used for the necessary correction. The burst size was calculated as the ratio of the number of phages produced to the number of infected cells.

CsCl buoyant equilibrium density gradient experiments

CsCl buoyant equilibrium density gradient experiments with P4 derivatives were performed as described by Nilssen *et al.* (1996). Portions of phage stocks (10^7 to 10^9 PFU in 50 to 100 μl) were mixed with 12 ml CsCl solution for which the average density was adjusted to 1.38 g/ml. Using 14 × 95 mm ultra clear Beckman tubes, centrifugation was carried out in a Beckman Ultracentrifuge (model LE-80K) at 55,000 × g (SW 41.1 Ti rotor at 21,000 rpm), for 60 h at 4°C. After the spin, the tubes were punctured at the bottom and 28 to 30 fractions (the volume of each fraction was about 0.43 ml) were collected. The refractory index of each fraction was determined using an Abbe-refractometer (Atigo model DR-A1, Japan) and converted to the density of the CsCl solution by the equation: $\rho = 10.2402 \times \eta - 12.6483$, where ρ is the density at 25°C and η is the refractory index of each fraction. Each fraction was titrated for P4 PFUs using C353 as an indicator.

Results and Discussion

Plasmid P4 ash8 delRI:: kmr and P4 ash8 delRI:: apr construction

The *ash8* (adapted to a second helper phage P3) mutation of P4 is a point mutation in the presumed repressor gene *cI*. It was isolated as a plaque former with second helper phage P3 and enables P4 to be stably maintained as a plasmid in the absence of helper phage (Lin, 1984). Many plasmid type P4 derivatives have been constructed with P4 *ash8* as the starting material for its plasmid maintenance characteristics.

In order to construct the plasmid P4 derivative, we removed the nonessential region of P4 *ash8* plasmid DNA by complete digestion with *Eco*RI. The resulting shortened P4 *ash8* lost the P4 DNA segment between the *Eco*RI site at nucleotide number 220 and the *Eco*RI site at nucleotide number 3633 of the P4 sequence (Ziermann *et al.*, 1993). This DNA segment contains the *gop*, *beta* and part of *int* genes which have been known to be nonessential for the bacteriophage type multiplication of P4 (Fig. 1A) (Ghisotti *et al.*, 1990). A kanamycin resistance (*kmr*) cassette obtained from pUC4K by *Eco*RI cleavage was introduced into the shortened P4 *ash8* to give it the selection marker. The transformation of *E. coli* C1a with the ligated mixture of shortened P4 *ash8* and *kmr* cassette yielded kanamycin resistant colonies. With restriction enzyme digestion analysis of these transformants, we confirmed the construction of P4 *ash8 del*RI:: *kmr*. The size of P4 *ash8 del*RI:: *kmr* was calculated as 9,495 bp (Fig. 1B). Regarding the inserted *kmr* gene orientation, two different types of P4 *ash8 del*RI:: *kmr* were possible. The *Sma*I site in

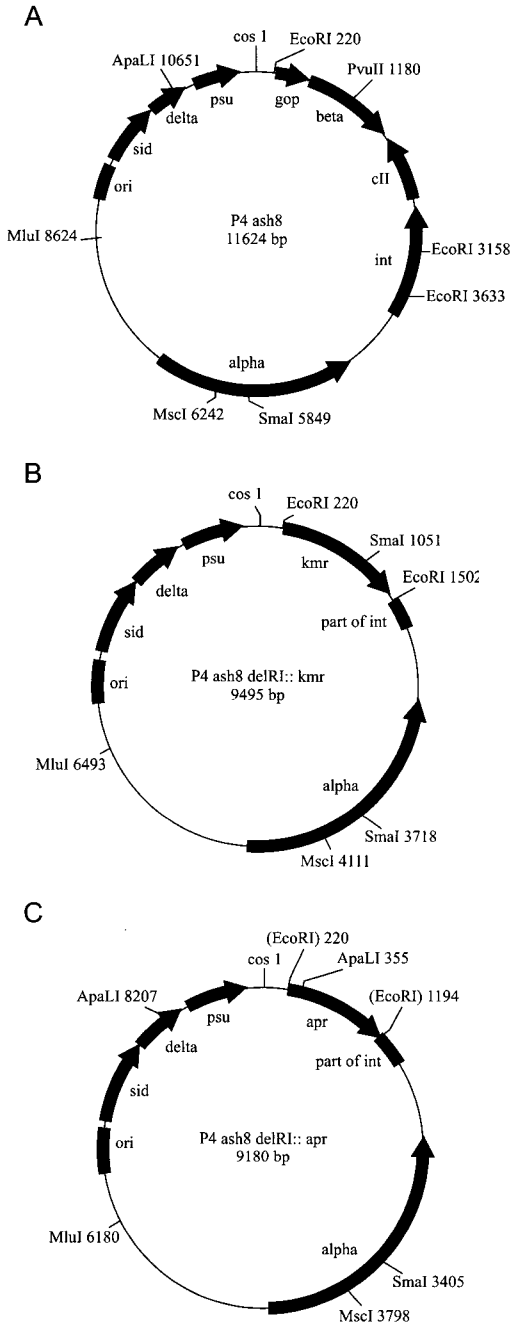

Fig. 1. Circular genetic maps of P4 *ash8* (A), P4 *ash8 del*RI:: *kmr* (B) and P4 *ash8 del*RI:: *apr* (C). The enzyme sites used in this study are shown. The positions of restriction sites are given in bp according to the P4 sequence (GenBank accession number X51522) in (A). Some genes and *ori* of P4 are also shown. In (B), the fragment from *Eco*RI at 220 to *Eco*RI at 3,633 of P4 DNA sequence was swapped with *kmr* of pUC4-K. The *Eco*RI at 1,502 in (B) corresponds to the *Eco*RI at 3,633 of the P4 DNA sequence. In (C), the fragment from *Eco*RI at 220 to *Eco*RI at 3,633 of the P4 DNA sequence was swapped with *apr* of Litmus38. Both *Eco*RI sites of P4 were destroyed during construction, so the positions of ligation are shown as (*Eco*RI) at 220 and 1,194 of P4 *ash8 del*RI:: *apr*. The (*Eco*RI) at 1,194 in (C) corresponds to the *Eco*RI at 3,633 of the P4 DNA sequence.

kmr helped to determine the *kmr* orientation. We isolated two kinds of P4 *ash8 del*RI:: *kmr*; the one with the opposite orientation of *kmr* shown in Fig. 1B was named as P4 *ash8 del*RI:: *kmr*R.

The construction of P4 *ash8 del*RI:: *apr* was basically the same as that of P4 *ash8 del*RI:: *kmr*. *Eco*RI digested plasmid P4 *ash8* DNA was treated with S1 nuclease to make both ends blunt. The resulting shortened P4 DNA was ligated with ampicillin resistance (*apr*) gene segment obtained from plasmid Litmus38 by *Ssp*I (blunt-end cutter) cleavage. Ampicillin resistant transformants were selected and analyzed with restriction enzyme digestion. We confirmed the construction of P4 *ash8 del*RI:: *apr*. The calculated size of P4 *ash8 del*RI:: *apr* was 9,180 bp (Fig. 1C). Regarding the inserted *apr* gene orientation, two different types of P4 *ash8 del*RI:: *apr* were possible. The *Apa*LI site in *apr* helped to determine the *apr* orientation. We isolated two kinds of P4 *ash8 del*RI:: *apr*; the one with the opposite orientation of *apr* shown in Fig. 1C was named as P4 *ash8 del*RI:: *apr*R.

Recovery of bacteriophage P4 derivatives

As plasmid P4 *ash8 del*RI:: *kmr*(R) and P4 *ash8 del*RI:: *apr*(R) have the *ori* and *cos* sites, they can be multiplied as bacteriophages in the presence of helper phage P2 (Bertani and Six, 1988). The sizes of their genomes were shorter than that of P4 *del22* (about 9,900 bp), which was the smallest P4 derivative packaged into P4-size head before this study (Raimondi *et al.*, 1985). This raises the question of whether the genome of P4 *ash8 del*RI:: *kmr* and P4 *ash8 del*RI:: *apr* can be packaged into the P4-size head.

To produce the bacteriophage version of the shortened P4 derivatives, we infected the lawn of *E. coli* C1a carrying plasmid P4 *ash8 del*RI:: *kmr*(R) or P4 *ash8 del*RI:: *apr*(R) with helper phage P2 *vir1*. The plaque that appeared on the lawn was taken and dissolved in LB as a phage solution. Each phage solution was tested against P2 and P4 indicator strains (Kim, 1998) and found to be bacteriophage P4-like due to their ability to form plaques only on the P4 indicator strain, *E. coli* C353. This suggested that the shortened P4 genome could be packaged into the P4-size head.

Starting from a single plaque, we prepared the phage stocks of shortened P4 derivatives. Those phage stocks could transduce *E. coli* C1a cells to kanamycin or ampicillin resistant transductants. Plasmid DNAs isolated from transductants were analyzed with restriction enzyme digestion and were confirmed to be the plasmids P4 *ash8 del*RI:: *kmr*(R) or P4 *ash8 del*RI:: *apr*(R) (Fig. 2). A transduction experiment showed that the P4 derivative genome in the phage head was biologically active.

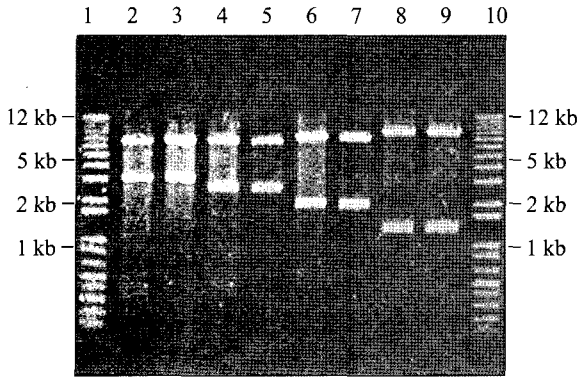

Fig. 2. Restriction enzyme digestion analysis of plasmid type P4 derivatives and the plasmid isolated from their transductants. Lane 1, 1 kb DNA ladder, Lane 2, P4 *ash8 del*RI:: *kmr*R digested with *Sma*I, Lane 3, plasmid DNA from P4 *ash8 del*RI:: *kmr*R transductant digested with *Sma*I, Lane 4, P4 *ash8 del*RI:: *kmr* digested with *Sma*I, Lane 5, plasmid DNA from P4 *ash8 del*RI:: *kmr* transductant digested with *Sma*I, Lane 6, P4 *ash8 del*RI:: *apr*R digested with *Apa*LI, Lane 7, plasmid DNA from P4 *ash8 del*RI:: *apr*R digested with *Apa*LI, Lane 8, P4 *ash8 del*RI:: *apr* digested with *Apa*LI, Lane 9, plasmid DNA from P4 *ash8 del*RI:: *apr*R digested with *Apa*LI, Lane 10, 1 kb DNA ladder.

Table 1. Heat stability of P4 and its derivatives

Phage	Stability[a]
P4 *ash8*	0.05 ± 0.01
P4 *ash8 del*RI:: *kmr*	1.16 ± 0.22
P4 *ash8 del*RI:: *kmr*R	0.95 ± 0.15
P4 *ash8 del*RI:: *apr*	0.94 ± 0.25
P4 *ash8 del*RI:: *apr*R	0.88 ± 0.04

[a]Heat stability was calculated as the ratio of viable phage before and after heat treatment in SSC buffer at 45°C. All heat stability data is the mean ± standard deviation of more than three independent experiments.

Table 2. Burst size and genome size of P4 and its derivatives

	Burst size[a]	Genome size (bp)
P4 *ash8*	63.3 ± 10.9	11,624
P4 *ash8 del*RI:: *kmr*	51.6 ± 10.5	9,495
P4 *ash8 del*RI:: *kmr*R	52.2 ± 7.1	9,495
P4 *ash8 del*RI:: *apr*	31.9 ± 1.8	9,180
P4 *ash8 del*RI:: *apr*R	34.1 ± 6.9	9,180

[a]All burst size data is the mean ± standard deviation of more than three independent experiments.

Heat stability of bacteriophage P4 derivatives

The P4 derivative phages recovered in this study had shortened P4 genomes containing antibiotic markers. These were a kind of artificial P4 deletion mutant. Deletion mutants of bacteriophages have been known to be resistant to high temperatures in relatively high salt concentrations (Parkinson and Huskey, 1971; Bertani and Chattoraj, 1980). Although the exact mechanism of this phenomenon has not yet been elucidated, many deletion mutants of lambda, P2, and P4 were isolated as phages surviving prolonged incubation in SSC (0.15 M NaCl plus 0.015 M sodium citrate) solution at high temperature (45°C) and showed heat stability.

To verify whether the artificial P4 derivative phages, P4 *ash8 del*RI:: *kmr*(R) and P4 *ash8 del*RI:: *apr*(R), are deletion mutants, we tested their heat stability. The number of viable phages before and after heat treatment in SSC buffer was counted and their ratio was a measure of the P4 derivatives' heat stability (Table 1). Four different shortened P4 derivatives appeared more stable than P4 *ash8* under these experimental conditions. The orientation of the inserted antibiotic markers did not affect the stability (Table 1). The heat stability of the shortened P4 derivatives was nearly 100%, indicating that the artificial P4 derivatives constructed and recovered in this study were deletion mutants of P4 phage.

Characterization of bacteriophage P4 derivatives

To characterize the bacteriophage P4 derivatives, their burst sizes were determined with a one-step growth experiment. Table 2 shows the burst sizes for P4 *ash8* and shortened P4 derivatives with wild type P2 lysogen (*E. coli* C295). The burst sizes for P4 *ash8 del*RI:: *kmr*(R) and P4 *ash8 del*RI:: *apr*(R) were smaller than that of P4 *ash8*. There was no difference in burst size between P4 *ash8 del*RI:: *kmr* and P4 *ash8 del*RI:: *kmr*R. The burst size of P4 *ash8 del*RI:: *apr* was nearly the same as that of P4 *ash8 del*RI:: *apr*R. Comparing the burst size of P4 *ash8* with that of P4 *ash8 del*RI:: *kmr*(R) and P4 *ash8 del*RI:: *apr*(R), it was evident that the reduction in genome size decreased the burst size. The decrease of burst size in P4 derivatives is likely to be due to the inefficiency of packaging of the small P4 derivative genome into the P4-size head.

Raimondi *et al.* (1985) suggested that the minimum size for a genome to be packageable into the P4-size head is 9.5 kb, which is equivalent to 82% of the P4 genome. In fact, no viable P4 derivative phage whose genome was smaller than P4 *del22* (9,975 bp) had been isolated until now. In this work, we isolated P4 derivative phages with genomes shorter than 9.5 kb and their burst sizes were much smaller than that of wild type P4 *ash8*. In the case of P4 *ash8 del*RI:: *apr*(R), the genome size was 79% of the P4 genome and the burst size was 50% of that of P4 *ash8*.

CsCl buoyant equilibrium density gradient experiments of P4 derivatives

To confirm the packaging of P4 derivatives into the P4-size head, we analyzed P4 *ash8 del*RI:: *kmr* and P4 *ash8 del*RI:: *apr* phage stocks with the CsCl buoyant equilibrium density gradient ultracentrifugation. P4 *ash8* was included as a control for the phage particle containing wild type P4 genome inside the small P4-size head. The phage stocks of three kinds of P4 were treated as described in Materials and Methods. Fractions were collected after ultracentrifugation. Each fraction was titrated for the viable phages and the refractory index was measured. The density profile was attained by plotting the plaque forming units of each fraction against its density.

Fig. 3 shows the profiles for P4 *ash8* (wild type) and P4 derivatives. Each profile exhibited a peak in plaque forming units at a different density. In the profile of P4 *ash8*, the peak was at a density of 1.3572 (Fig. 3A). This value corresponds to the published density of P4 wild type phage particle (Shore *et al.*, 1978). The peak for P4 *ash8 del*RI:: *kmr*'s profile was at a density of 1.3521, which was smaller than that of P4 *ash8* (Fig. 3B). The reduction of density in P4 *ash8 del*RI:: *kmr*'s peak resulted from the smaller genome of P4 *ash8 del*RI:: *kmr* compared to P4 *ash8*. As expected, P4 *ash8 del*RI:: *apr,* whose genome was the shortest of the three, had the smallest peak density, 1.3460 (Fig. 3C). The peak in each profile confirms not only the packaging of shortened P4 plasmid DNA into the P4-size head but also the genome size of the shortened P4 derivative relative to its density.

The *cos*-cleavage reaction of the bacteriophage P2-P4 system is different from that of other lambdoid phage systems in its preference for substrate DNA conformation. The covalently closed circular form of P2 or P4 DNA is the preferred substrate in the bacteriophage P2-P4 system (Bertani and Six, 1988). For the *in vitro cos*-cleavage and packaging reaction of the bacteriophage P2-P4 system, a more easily obtainable P4 plasmid derivative is needed as a substrate molecule. The P4 derivatives constructed in this study will be good substrate molecules because they have antibiotic markers for easy selection and can be packaged into the P4-size head efficiently.

In conclusion, we constructed P4 derivative plasmids having the smallest genome size packageable into the small P4-size head *in vitro* and recovered them as biologically active phage particles.

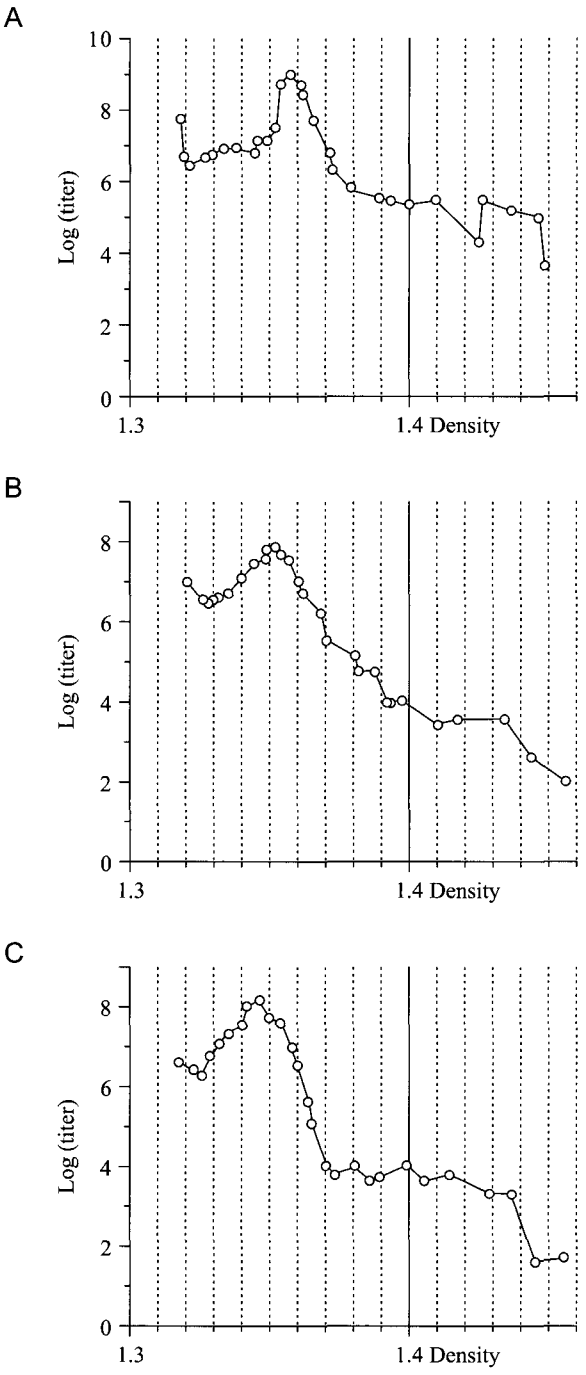

Fig. 3. CsCl buoyant equilibrium density gradient profiles of P4 *ash8* (A), P4 *ash8 del*RI:: *kmr* (B) and P4 *ash8 del*RI:: *apr* (C). The ordinate of these profiles shows the P4 titer of each fraction expressed in log scale, and the abscissa shows the density of each fraction measured with a refractometer.

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