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보 문

Construction and characterization of the bacteriophage P4 derivatives whose genome size suitable for packaging into a P2$_{sir3}$-sized head

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P2 $_{sir3}$-sized head에 packaging 되기 용이한 크기의 박테리오파지 P4 유도체 조성과 정성 연구

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ABSTRACT: The term "P2 *sir*-associated helper inefficiency" has been used to define the inefficient helper capability of P2 *sir* mutants for their satellite bacteriophage P4. The aim of this study was to investigate the factors overcoming P2 *sir*-associated helper inefficiency. At first, we verified whether the P2 *cos* region containing P4 *sid71 cosP2* could overcome P2 *sir*-associated helper inefficiency with P2 *sir3*. The result was that P4 *sid71 cosP2* could not overcome P2 *sir*-associated helper inefficiency with P2 *sir3*. Instead of *cos* region of P2, the size of the DNA packaged into a P2$_{sir}$-sized head seems to be important for overcoming P2 *sir*-associated helper inefficiency. In the present work, three kinds of P4 derivatives with packaged DNA sizes between those of P4 *ost1* and P4 *ost2*, were constructed through DNA manipulation. In one P4 derivative, P4 *sid71 delRI::apr*, the size of the packaged DNA was identified with a CsCl buoyant equilibrium density gradient experiment. According to the burst sizes of the P4 derivatives, they could overcome P2 *sir3*-associated helper inefficiency. The size of the P4 derivative DNA suitable for packaging into a P2$_{sir3}$-sized head was 28-29 kb.

Key words: *Escherichia coli*, P2 *sir*, bacteriophage P2-P4, burst size, packaging

Bacteriophage P4 has a unique life cycle. It is a defective phage, having no viral head and tail genes (Six and Klug, 1973). It can only multiply as a phage when a helper phage, such as bacteriophage P2 or phage 186, is present in the same host cell. In the absence of a helper phage, it can be maintained as a plasmid (Bertani and Six, 1988). Although P4 needs P2 for its head and tail synthesis, the DNA sequences of P4 and P2 show no homology, except for a 19 bp-long cohesive (*cos*) end site (Bertani and Six, 1988). As the genome size of P4 (11,624 bp-long) is roughly one third that of P2 (33,574 bp-long), P4 requires a viral head smaller than that of P2. P4 possesses a *sid* (size determination) gene to enable P2 to assemble a small viral head suitable for P4's genome (Barrett *et al.*, 1976).

Genetic studies of P2 and P4 isolated *sid*$^-$ mutants of P4 that assembled a large, P2-sized, head with a P2 helper phage (Shore *et al.*, 1978). Some of the isolated P2 mutants had the ability to assemble a large head in the presence of the *sid* gene of P4. Those mutations were named *sir* (*sid* responsiveness) and mapped in the middle of the *N* gene of P2 that codes for the major capsid protein of the viral head (Six *et al.*, 1991). As P2 *sir* mutants always assemble a large head, it was assumed that P2 *sir* mutants would be good helpers for a P4 *sid*$^-$ mutant phage. However, burst size experiments showed that the burst size of P4 *sid*$^-$ with a P2 *sir* helper was 10-20 times smaller than that of P4 *sid*$^-$ with a P2 wild-type helper (Six *et al.*, 1991). Thus, P2 *sir* cannot efficiently act as a helper phage for P4. This

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phenomenon has been called P2 *sir*-associated helper inefficiency (Kim, 2003).

To investigate P2 *sir*-associated helper inefficiency, two P4 derivatives were isolated as plaque formers with P2 *sir3* which is the most inefficient P2 *sir* in terms of helper capability for P4: P4 *ost1* (overcome sir three) and P4 *ost2* (Kim *et al.*, 1998; Kim, 2003). Characterization of these P4 *ost* mutants showed that the number of *cos*- sites did not affect the Ost phenotype. Instead, the genome size of the packaged DNA in the $P2_{sir}$-sized head appeared to be a key factor in P2 *sir*-associated helper inefficiency. The genome sizes of both *ost* mutants of P4 are between two copies (dimeric) of the P4 genome size and three copies (trimeric) of the P4 genome size (Kim, 2003).

The *cos* region of P2 and P4 was defined as about a 250 bp-long DNA sequence around the *cos*-site (Ziermann and Calendar, 1990). The dissimilarity of the *cos* region of P2 and P4 was previously reported (Kim, 2013). When the P4 *cos* region of P4 *sid71* was replaced with the P2 *cos* region to construct a *sid*$^-$ P4 containing *cos* region of P2, the resulting P4 *sid71 cosP2* overcame P2 *sir*-associated helper inefficiency with P2 *sir2* showing strong Sir phenotype (Kim, 2013). Thus, the *cos* region of P2 seems to be responsible for P2 *sir*-associated helper inefficiency especially with P2 *sir2*. As reported previously, the temperature sensitivity and extreme P2 *sir*-associated helper inefficiency of the *sir3* mutation is slightly different from those of other *sir* mutations (Six *et al.*, 1991). Therefore, it is not known, whether the *cos* region of P2 is a key factor in overcoming P2 *sir*-associated helper inefficiency with all *sir* mutants of the P2 helper.

In this study, using a burst size study of P4 *sid71 cosP2* with the P2 *sir3* helper, we verified whether the P2 *cos* region containing P4 *sid*$^-$ could overcome P2 *sir*-associated helper inefficiency, with the P2 *sir3* helper. We then constructed P4 derivatives with packaged genome size between that of the dimeric P4 genome and trimeric P4 genome and tested whether they could overcome P2 *sir*-associated helper inefficiency, with the P2 *sir3* helper.

Materials and Methods

Bacterial strains, bacteriophages, and plasmids

The *Escherichia coli* C1a strain 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 (Six and Klug, 1973). $P2^+$ lysogen (*E. coli* C295), P2 *sir2* lysogen (*E. coli* C2142), and P2 *sir3* lysogen (*E. coli* C2143) were used for the burst size determination (Six *et al.*, 1991). The bacteriophages used in this study are listed in Table 1.

The plasmids Litmus38 (NEB, USA) and pUC4-K (Norrander *et al.*, 1983) were used as sources of the ampicillin resistance (*apr*) gene and kanamycin resistance (*kmr*) gene cassette, respectively.

Media

Luria Bertani (LB) broth or LB agar were used as the culture media in this study. Top soft agar (7 g of trypton, 5 g of NaCl,

Table 1. Bacteriophage P2, P4 and P4 derivatives used in this study

	Description	References
P2 *vir1*	*C* (repressor) gene defective	Bertani and Six (1988)
P4	Wild type	Six and Klug (1973)
P4 *sid101*	*sid* gene deficient	Nilssen *et al.* (1996)
P4 *sid71*	*sid* gene deficient	Kim *et al.* (1998)
P4 *ost1*	Can form plaque with P2 *sir3* lysogen	Kim *et al.* (1998)
P4 *ost2*	Can form plaque with P2 *sir3* lysogen	Kim (2003)
P4 *sid71 cosP2*	P4 *sid71* containing *cos* region of P2	Kim (2013)
P4 *sid71 delRI::apr*	Non-essential P4 DNA deleted, Ap^R	This work
P4 *sid71 delRI::kmr*	Non-essential P4 DNA deleted, Kan^R	This work
P4 *sid101 delRI::kmr*	*sid* gene deficient Non-essential P4 DNA deleted, Kan^R	This work

and 7 g of agar per L) was used for the overlay pouring. LB supplemented with anti-P2 serum (neutralization constant k = 1/min) (LBSe) was used in one-step growth experiments. If antibiotic selection was needed, ampicillin or kanamycin (50 µg/ml) was added to the LB broth or LB agar. The incubation temperature was 37℃, unless otherwise noted.

DNA manipulation

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

Recombinant DNA techniques were carried out according to Sambrook and Russel (2001).

Construction of P4 *sid71 delRI::apr*

The plasmid DNA of P4 *sid71* was digested with *Eco*RI to produce an 8.2 kb-long fragment (from *Eco*RI at nucleotide number 3,633 of the P4 DNA sequence through *cos* 1 to *Eco*RI at nucleotide number 220 of the P4 DNA sequence in Fig. 1). This DNA fragment was treated with S1 nuclease to make both ends blunt. For the *apr* gene, plasmid Litmus38 DNA was cleaved with *Ssp*I (blunt-end cutter). The resulting 973 bp-long fragment harbored an *apr* gene. A derivative, presumed to be P4 *sid71 delRI::apr*, was constructed through the ligation of both fragments. Ampicillin-resistant colonies were selected by DNA transformation with *E. coli* C1a competent cells. Restriction enzyme digestion analysis of the transformants confirmed that the derivative was P4 *sid71 delRI::apr.*

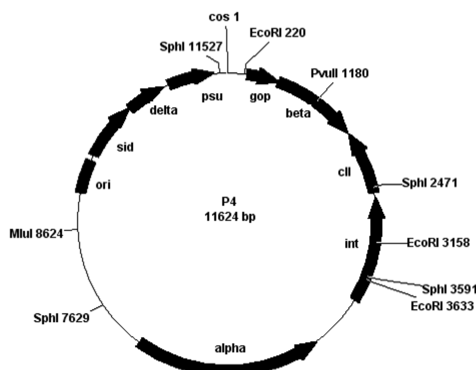

Fig. 1. The genetic and physical map of plasmid P4. The position of the restriction enzyme sites are given in bp according to the P4 sequence (GenBank accession number; X51522). Some genes and the origin of replication (*ori*) are shown.

Construction of P4 *sid71 delRI::kmr*

Plasmid DNA of P4 *sid71* was digested with *Eco*RI to produce an 8.2 kb-long fragment (from *Eco*RI at nucleotide number 3,633 of the P4 DNA sequence through *cos* 1 to *Eco*RI at nucleotide number 220 of the P4 DNA sequence in Fig. 1). For the *kmr* gene cassette, plasmid pUC4-K was cleaved with *Eco*RI. The resulting 1.24 kb-long *kmr* gene cassette was ligated with the 8.2 kb-long P4 *sid71* segment to obtain a derivative presumed to be P4 *sid71 delRI::kmr*. Kanamycin-resistant colonies were selected by DNA transformation with *E. coli* C1a competent cells. Restriction enzyme digestion analysis of the transformants confirmed that the derivative was P4 *sid71 delRI::kmr.*

Construction of P4 *sid101 delRI::kmr*

The *sid101* mutation is a 195 bp deletion within the *sid* gene that does not change the *sid* reading frame (Nilssen *et al.*, 1996). The plasmid DNA of P4 *sid101* was digested with *Eco*RI to produce an 8.0 kb-long fragment (from *Eco*RI at nucleotide number 3,633 of the P4 DNA sequence through *cos* 1 to *Eco*RI at nucleotide number 220 of the P4 DNA sequence in Fig. 1). For the *kmr* gene cassette, plasmid pUC4-K was cleaved with *Eco*RI. The resulting 1.24 kb-long *kmr* gene cassette was ligated with the 8.0 kb-long P4 *sid101* segment to obtain a derivative presumed to be P4 *sid101 delRI::kmr*. Kanamycin-resistant colonies were selected by DNA transformation with *E. coli* C1a competent cells. Restriction enzyme digestion analysis of the transformants confirmed that the derivative was P4 *sid101 delRI::kmr.*

Bacteriophage stock preparation

Bacterial lawns of *E. coli* C1a harboring the constructed P4 derivatives were spotted with one drop of helper bacteriophage, P2 *vir1*, and incubated at 37℃ overnight. The next morning, an area of lysis appeared on the spot. Agar plugs were taken from the lysis area and transferred to 1 ml of LB. This phage suspension was plated on an *E. coli* C353 lawn as a P4 indicator to obtain a single plaque. The preparation of bacteriophage P4 derivative stock was performed according to Kim and Song (2006).

CsCl buoyant equilibrium density gradient experiments

The CsCl buoyant equilibrium density gradient experiments were performed as described by Nilssen *et al.* (1996). Portions of phage stocks were mixed with 12 ml of CsCl solution, with the average density adjusted to 1.38 g/ml. The mixture in a 14 × 95 mm ultra clear Beckman tube was centrifuged 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℃. After centrifugation, the tube was punctured at the bottom, and 28 to 30 fractions (the volume of each fraction was 0.4 ml) were collected. The refractory index of each fraction was measured using an Abbe refractometer (Atigo model DR-A1) and converted to the density of the CsCl solution. Each fraction was titrated for P4 plaque-forming units (PFUs) using C353 as a P4 indicator. The profile was obtained by plotting the PFUs of each fraction against its density.

One-step growth experiments

One-step growth experiments were done according to Kim and Song (2006). The burst size was calculated as the ratio of the number of phages produced to the number of infected cells.

Results and Discussion

A previous study showed that the replacement of the P4 *cos* region with a P2 *cos* region in P4 *sid71* rendered P4 *sid71 cosP2* able to overcome P2 *sir*-associated helper inefficiency with a P2 *sir2* helper (Kim, 2013). The present study determined whether the *cos* region of P2 in P4 *sid71 cosP2* could overcome P2 *sir*-associated helper inefficiency with a strong P2 *sir3* helper. Analysis of the burst size of P4 *sid71 cosP2* with the P2 *sir3* lysogen revealed that the burst size was more than 14 times less than that with the P2 *sir2* lysogen (Table 2). Although *sir3* is a unique *sir* mutation, in that it confers extreme helper inefficiency and temperature sensitivity (Six *et al*., 1991), the *cos* region of P2 was not sufficient to overcome P2 *sir*-associated helper inefficiency with any P2 *sir* lysogen, especially the P2 *sir3* lysogen. Thus, it appears that the reported ability of P4 *sid71 cosP2* to overcome P2 *sir2*-associated helper inefficiency is a special case.

Table 2. **Burst size of P4 and P4 *sid71 cosP2***

Phage	Burst size with C1a lysogenic for[a]		
	P2^{+}	P2 *sir2*	P2 *sir3*
P4	111±4	1.4±0.4	0.2±0.04
P4 *sid71 cosP2*	46±11	52±9	3.7±0.7

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

Previous studies reported that P4 *ost1* and P4 *ost2*, isolated derivatives of P4, overcame P2 *sir*-associated helper inefficiency with P2 *sir3* (Kim *et al.*, 1998; Kim, 2003). It was suggested that the number of *cos* sites in P4 derivatives and the packaged DNA size in P2$_{sir}$-sized head were important to overcome P2 *sir*-associated helper inefficiency (Kim *et al.*, 1998). However, a later P4 *ost2* study demonstrated that the number of *cos* sites was not important (Kim, 2003). Thus, only the packaged DNA size in the P2$_{sir}$-sized head seems to be important. Based on the packaged DNA sizes of P4 *ost1* (26.6 kb) and P4 *ost2* (28.8 kb), the effective size was thought to be somewhere between a dimeric P4 genome size (23.2 kb) and a trimeric P4 genome size (34.8 kb).

In this study, several P4 derivatives with packaged DNA between the genome size of P4 *ost1* and that of P4 *ost*2 were constructed to clarify whether the packaged DNA size affected P2 *sir*-associated helper inefficiency. Three P4 derivatives were constructed: P4 *sid71 delRI::apr*, P4 *sid71 delRI::kmr*, and P4 *sid101 delRI::kmr.* All had the *sid* mutation and were shortened by deleting non-essential genes (*gop*, *beta*, *cII*, and *int*) with *Eco*RI digestion (see Fig. 1). In the case of the *sid101* mutation, the *sid* gene has a 195 bp-long in-frame deletion (Nilssen *et al.*, 1996). Antibiotic-resistant markers were introduced by inserting an ampicillin-resistant gene (*apr*) or kanamycin-resistant gene cassette (*kmr*). The packaged DNA size into the P2$_{sir}$-sized heads of these derivatives was 27.5 kb (for P4 *sid71 delRI::apr*), 27.9 kb (for P4 *sid101 delRI::kmr*), and 28.5 kb (for P4 *sid71 delRI::kmr*). Their packaged DNA sizes were between that of P4 *ost1* and P4 *ost2*.

One of the P4 derivatives, P4 *sid71 delRI::apr*, was analyzed with a CsCl buoyant equilibrium density gradient experiment to confirm that three copies of its genome (trimeric DNA) were packaged into the P2-sized head. The CsCl buoyant equilibrium density gradient profiles of P4 *sid*71 and P4 *sid71 delRI::apr*

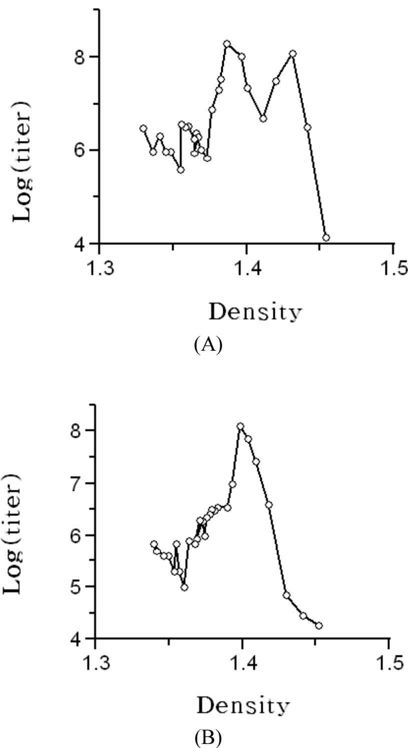

Fig. 2. CsCl buoyant equilibrium density gradient profiles of P4 *sid71* stock (A); P4 *sid71 delRI::apr* stock. The ordinates of the profiles show the P4 titer of each fraction expressed in log scale, and the abscissas show the density of each fraction measured with a refractometer.

are shown in Fig. 2. In the profile of P4 *sid71*, two peaks having nearly the same height appeared at the density corresponding to two copies of the P4 genome packaged into the P2-sized head and at the density corresponding to three copies of the P4 genome packaged into the P2-sized head. In contrast, in the profile of P4 *sid71 delRI::apr*, only one peak appeared at the density between two peaks of P4 *sid71*. The density corresponded to the density of three copies of the P4 *sid71 delRI::apr* genome packaged into the P2-sized head. The findings confirmed that the newly constructed P4 derivatives packaged into the P2-sized head had a trimeric form of DNA (or three copies of the genome together).

The burst sizes of the newly constructed P4 derivatives, P4 *ost1*, P4 *ost2*, P4 *sid101,* and P4 *sid71* were determined with wild-type P2[+] lysogen, P2 *sir2* lysogen, and P2 *sir3* lysogen hosts. The results are summarized in Table 3. All the newly constructed P4 derivatives overcame P2 *sir*-associated helper inefficiency with each P2 *sir* lysogen. The results suggested that the size of the packaged DNA is important in overcoming

Table 3. Characterization of P4 derivatives by one-step growth experiments

Phage	Packaged DNA (kb)	Burst size with C1a lysogenic for[a]		
		P2[+]	P2 *sir2*	P2 *sir3*
P4 *ost1*	26.6	91±2	93±4	22±3
P4 *sid71 delRI::apr*	27.5[b]	62±2	69±4	32±4
P4 *sid101 delRI::kmr*	27.9[b]	99±4	74±5	41±3
P4 *sid71 delRI::kmr*	28.5[b]	75±3	48±2	38±4
P4 *ost2*	28.9	105±4	144±3	35±4
P4 *sid101*	22.9/34.3[c]	39±2	10±1	4±0.3
P4 *sid71*	23.3/34.9[c]	41±3	4±0.5	2±0.2

[a] All the burst size data are the mean±standard deviation of more than three independent experiments.
[b] Three copies of genomic DNA (trimeric DNA) were packaged into a large P2-sized head.
[c] Two copies of genomic DNA (dimeric DNA) or three copies of genomic DNA (trimeric DNA) were packaged into a large P2-sized head.

Fig. 3. Size dependence of phage production for P4 derivatives packaged into the P2$_{sir3}$-sized head. The ordinate shows the burst size of each P4 derivative, and the abscissa shows the size of the DNA packaged into each P4 derivatives'. The burst size of each P4 derivative was determined by a one-step growth experiment. The burst size and the size of each P4 derivative were taken from Table 3.

P2 *sir*-associated helper inefficiency. They also indicate that any P4 derivatives with packaged DNA between 26 kb and 29 kb in a P2-sized head should be able to overcome P2 *sir*-associated helper inefficiency even with P2 *sir3*.

To investigate the relationship between the size of the packaged DNA and the Ost (overcome *sir* three) phenotype, the sizes of the packaged DNA and the burst sizes with the P2 *sir3* lysogen of the P4 derivatives in Table 3 were plotted (see Fig. 3). In the plot, the peak appeared at around 28-29 kb. This implies that the inner volume of a P2$_{sir3}$-sized head might be suitable for packaging a 28–29 kb -long DNA molecule.

A previous study demonstrated that in lambda phages, mutations in the major capsid protein gene *E* altered the size of the head and the size of packaged DNA (Katsura, 1983). The

sir3 mutation is a point mutation in the middle of the major capsid protein gene *N* of P2 (Six *et al.*, 1991). It causes non-conservative replacement (Met to Thr) and confers a severe temperature sensitive phenotype (Six *et al.*, 1991). P2 *sir3* has a low burst size (15) compared to that of the wild-type P2 (182) and other P2 *sir*, such as P2 *sir2* (251) (Six *et al.*, 1991). The aforementioned findings lead to the idea that the low burst size and helper inefficiency of satellite phage P4 of P2 *sir3* may be due to the smaller inner volume of the $P2_{sir3}$-sized head.

Bacteriophage P2 and P4 have a *cos* site and are packaged by a *cos*-cleavage packaging mechanism (Bertani and Six, 1988). When P2 *sir3* acts as a helper phage, the packaging seems to occur in a slightly different manner, with a specific size of DNA favored by the P2 $_{sir3}$-sized head. The possibility that P2 *sir3* favors a head-full DNA packaging mechanism should be studied further.

In conclusion, the *cos* region of P2 was unable to overcome P2 *sir*-associated helper inefficiency with P2 *sir3*. Through the construction and characterization of several P4 derivatives, the size of the DNA packaged into a $P2_{sir}$-sized head was shown to play a role in overcoming P2 *sir*-associated helper inefficiency with P2 *sir3*. The favorable size of DNA with a $P2_{sir3}$-sized head was around 28-29 kb.

적 요

“P2 *sir*-관련 도움파지 비효율성”이라는 용어는 P2 *sir* 변이체가 그들의 위성 박테리오파지인 P4에 대해 도움파지 역할을 충분히 못하는 것을 가리키는 용어이다. 이 연구의 목적은 이러한 P2 *sir*-관련 도움파지 비효율성을 극복하는 요인들을 조사하는 것이다. 우선 P2의 *cos* 영역을 함유하는 P4 *sid71 cosP2*가 P2 *sir3*의 도움파지 비효율성을 극복하는지를 조사하였다. 그 결과 P4 *sid71 cosP2*는 P2 *sir3*의 도움파지 비효율성을 극복하지 못하였다. P2의 *cos* 영역 대신에 $P2_{sir}$-sized head에 packaging되는 DNA 크기가 도움파지 비효율성을 극복하는 중요한 요인으로 나타났다. 이 연구에서는, DNA 조작을 통해 packaging 되는 DNA 크기가 P4 *ost1*과 P4 *ost2* 사이가 되는 세 종류의 P4 유도체를 조성하였다. 그 중 하나인 P4 *sid71 delRI::apr*의 CsCl 균등밀도차실험을 거쳐 packaging되는 DNA의 크기를 확인하였다. P4 유도체들의 후손방출량 실험에 따르면 그들은 모두 P2*sir3*-관련 도움파지 비효율성을 극복하였다. $P2_{sir3}$-sized head에 잘 packaging되는 P4 유도체의 크기는 28-29 kb로 나타났다.

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