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| 단 보 |

In Vitro Construction and Characterization of the Bacteriophage P4 Derivative, P4 *sid71 cos*P2, Containing the Bacteriophage P2 *cos* Region

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박테리오파지 P2의 *cos* 지역을 함유하는 박테리오파지 P4 유도체인 *P4 sid71 cos*P2의 *In vitro* 조성과 정성 연구

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Bacteriophage P2 *sir* mutants are inefficient helpers for their satellite bacteriophage P4. The term, "P2 *sir*-associated helper inefficiency" has been used to define this phenomenon and it has been suggested that the DNA sequence difference between the *cos* region of P2 and that of P4 is responsible. To test this hypothesis, P4 derivative phage, P4 *sid71 cos*P2, containing the *cos* region of P2 and *sid71* allele was constructed through several *in vitro* DNA manipulation steps. Its burst size was determined using a one-step growth experiment. The results showed that the substitution of the *cos* region of P2 for the *cos* region of P4 in P4 *sid71 cos*P2 overcame "P2 *sir*-associated helper inefficiency". P4 *sid71 cos*P2 stock phages prepared with P2 wild type helper and P2 *sir* helper were analyzed using a CsCl buoyant equilibrium density gradient experiment. The results revealed that the phage particles containing three copies of the P4 genome were the predominant particles in both cases.

***Keywords*:** *Escherichia coli*, bacteriophage P2-P4, *cos* region, packaging, P2 *sir*

Bacteriophage P4 is a satellite phage of the coliphage P2. It has no genes for virion synthesis and can be maintained as a plasmid in the absence of helper phage P2. However, bacteriophage P4 requires bacteriophage P2 as a helper phage for its lytic growth (Bertani and Six, 1988). The genome size of P2 is 33.5 kb long and the genome size of P4, 11.6 kb, is one-third of that of P2. The *sid* (size determination) gene of P4 was isolated due to its ability to enforce P2 to assemble small head (Shore *et al*., 1978). The gpSid of P4, which forms an external scaffold around the P4-size prohead, enables P2 to assemble a P4-size head suitable for packaging the P4 genome (Marrvik *et al*., 1995; Dearborn *et al*., 2012). P4 *sid* mutants fail to assemble P4-size head. Two or three copies of the P4 *sid* genome are packaged into P2-size heads (Shore *et al*., 1978; Song and Kim, 2006). P2 mutants, which can assemble large P2-size heads in the presence of P4, have been isolated and called P2 *sir* (*sid* responsiveness) (Six *et al*., 1991). These *sir* mutations were identified in the *N* gene of P2, which encodes the capsid protein of P2. When P2 *sir* acted as a helper for P4, only P2-size heads were assembled and two or three copies of the P4 genome were packaged into those P2-size heads (Six *et al*., 1991).

When P4 *sid* infects the P2 *sir* lysogen host, we would expect vigorous production of P2-size head particles packaged with two or three copies of the P4 genome. But the actual production of progeny particles was inhibited, in this case (Six *et al*., 1991; Kim *et al*., 1998). This tells us that P2 *sir* cannot efficiently act as a helper phage for P4. The term, "P2 *sir*-associated helper inefficiency" has been coined to define this phenomenon (Kim, 2003).

Factors affecting P2 *sir*-associated helper inefficiency have been investigated to obtain information concerning the *cos*-cleavage and packaging mechanisms of the P2-P4 bacteriophage system. P4 *ost1* and P4 *ost2* were isolated and studied as suppressor mutants of P2 *sir3,* which showed a strong Sir phenotype (Kim *et al*., 1998; Kim, 2003). From the

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identification and characterization of these suppressor mutants, it was suggested that a key factor was the genome size of the packaged DNA in the $P2_{sir}$-size head. As P4 *ost1* and P4 *ost2* constitute an *in vivo* recombinant of a P4 multimer having a deletion, their genome size could not be compared with that of P4 packaged into a P2-size head, usually two copies of the P4 genome (23.2 kb) or three copies of the P4 genome (34.8 kb). Actually, the genome sizes of P4 *ost1* and P4 *ost2* are 26.6 kb and 28.8 kb, respectively (Kim *et al*., 1998; Kim, 2003). Therefore, P4 *ost1* and P4 *ost2* represent special cases for overcoming P2 *sir*-associated helper inefficiency.

Despite the P2 *sir*-associated helper inefficiency associated with P2 *sir* mutants, most P2 *sir* mutants, except P2 *sir3,* can apparently package their own DNA quite well (Six *et al*., 1991). This implies that the $P2_{sir}$-size head interacts differently with the P4 and P2 genomes during *cos*-cleavage and packaging. Ziermann and Calendar (1990) defined the *cos* region of P2 and P4 as a DNA sequence around the *cos*-site, which is necessary for *cos*-cleavage and packaging, by transduction experiments. The *cos* regions of P2 and P4 begin at about 120 bp upstream of the first nucleotide of the *cos*-site and end at the last nucleotide of the *cos*-site. They are composed of the *cos*-site or *cos* nicking site (*cosN*) and the binding sites for the terminase, possibly the prohead, and other proteins involved in packaging (*cosB*). Although the 19 bp-long *cos*-site of P4 is identical to that of P2, the DNA sequence surrounding the *cos*-site of P4 is not homologous to P2 (Ziermann and Calendar, 1990). The alignment of the DNA sequences including the *cos* regions of bacteriophages P2 and P4 is shown in Fig. 1. Conserved sequences appear from -143 to -137, -113 to -107, -102 to -97, -29 to -13 and -9 to -2. Conceivably, some of the sequence intervals for which P4 DNA differ from P2 DNA may be important for interaction with the $P2_{sir}$-size head and the low efficiency of P2 *sir* mutants as helpers for P4. It is assumed that the *cosB* sites of P4 differing from those of P2 may cause P2 *sir*-associated helper inefficiency. In the present study, this assumption was examined with the P4 (or *sid71*) derivative containing the *cos* region of P2 instead of the *cos* region of P4.

To obtain the DNA fragment containing the *cos* region of P2, the phage DNA of P2 was first isolated according to a previous method (Lindqvist, 1971). Isolated linear P2 phage DNA was circularized with a mild ligase treatment. A PCR reaction was performed with this circularized P2 phage DNA as a template using specific primers (P2BSP primer: 5′ -GGTGGTTCCGGATACGAAACGCTTT-3′ primer and P2XHO

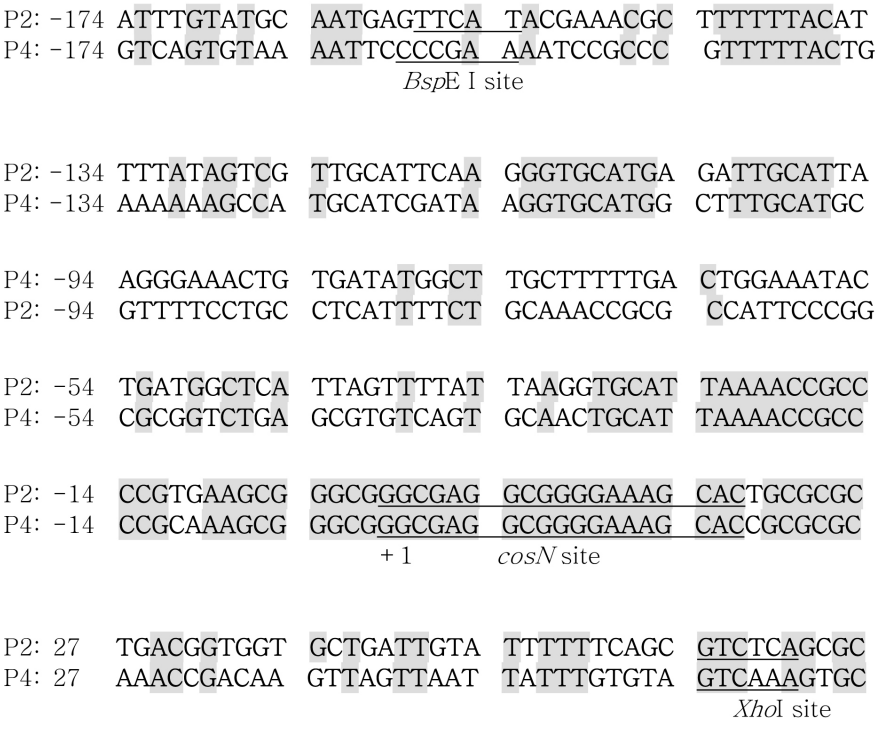

Fig. 1. Alignment of the *cos* regions of bacteriophages P2 and P4. Position number 1 indicates the G at the beginning of the left cohesive end found for the DNA of linearized phages. In the P2 and P4 sequences, the shading regions indicate the same nucleotides appearing in P2 and P4 sequences. The underlined *Bsp*EI and *Xho*I sites indicate the nucleotides where the restriction enzyme recognition sites were introduced by *in vitro* mutagenesis in this study.

primer: 5′-TGAAGGCACTCGAGGCACTTTG-3′ where the underlined sequences indicate the *Bsp*EI and *Xho*I restriction endonuclease recognition sequences, respectively). The resulting 220 bp-long amplified fragment was digested with both enzymes. Finally a "P2 *cos* region module," which has a *Bsp*EI site at one end and an *Xho*I site at the other end, was obtained.

P4 genomic DNA was isolated as a plasmid from a nonlysogenic *E. coli* C1a strain harboring P4 (Lin, 1984). The 1.2 kb *Bam*HI-*Eco*RI portion of the P4 DNA containing the *cos* region of P4 was ligated with the *Bam*HI/*Eco*RI digested pUC19 (NEB, USA) to construct a pKJK49 plasmid. The restriction endonuclease recognition sites, *Bsp*EI and *Xho*I, were introduced at -162 bp and at +57 bp of P4 sequences in pKJK49 by *in vitro* mutagenesis with the QuickChange™ Mutagenesis Kit (Stratagene, USA) for the P2 *cos* region module exchange. For the introduction of the *Bsp*EI site in pKJK49, a PCR reaction was performed with pKJK49 as a template using P4BSE (5′-AGTGTAAAATTCTCCGGAAAT CCGCCCGT-3′) and P4BSER (5′-ACGGGCGGATTTCCGG AGAATTTTACACT-3′) primers. According to the procedure in the QuickChange™ Mutagenesis Kit, the amplified fragments were digested with *Dpn*I to remove the template DNA. *E. coli* JM105 was transformed with that DNA to get a clone-harboring derivative pKJK49 with a *Bsp*EI site. For the introduction of an *Xho*I site, a PCR reaction was performed with the derivative pKJK49 with the *Bsp*EI site as a template using P4XHO (5′-CA AAGTGCCTCGAGTGCCTTCA-3′) and P4XHOR (5′-TGAA GGCACTCGAGGCACTTTG-3′) primers. Through *Dpn*I digestion and transformation, a pKJK49 plasmid having *Bsp*EI and *Xho*I recognition sites was constructed. The introduction of *Bsp*EI and *Xho*I recognition sites was identified by DNA sequencing.

The *cos* region replacement was performed between the *Bsp*EI/*Xho*I digested pKJK49 derivative and the P2 *cos* region module. The resulting plasmid was called pKJK76. The swapping of the *cos* region of P4 for the *cos* region of P2 in pKJK76 was confirmed by DNA sequencing.

P4 (or *sid71*) plasmid DNA was digested with two single cutters, *Sal*I (at nucleotide number 3,043 of the P4 DNA sequence) and *Mlu*I (at nucleotide number 8,624 of the P4 DNA sequence), and ligated with *Sal*I/*Mlu*I digested pGEM5Z (Promega, USA) to get two kinds of recombinant plasmids covering the whole P4 genome. The resulting two plasmids were called pKJK77 (or pKJK78 for the *sid71* allele) and pKJK100 (Fig. 2). The pKJK77(or pKJK78) had portions of P4 DNA starting at nucleotide number 8,624 of the P4 DNA sequence extending past *cos* and ending at nucleotide number 3,043 of the P4 DNA sequence. The pKJK77 (or pKJK78) insert contained late genes, such as *sid* (*sid71* allele in pKJK78), *delta* and *psu,* and the *cos* region. The pKJK100 insert had the remaining portion of the P4 DNA starting at nucleotide number 3,043 of the P4 DNA sequence and ending at nucleotide number 8,624 of the P4 DNA sequence.

An approximate 1.2 kb-long *Bam*HI-*Eco*RI fragment containing the *cos* region of P2 in pKJK76 was taken and replaced with the *Bam*HI-*Eco*RI fragment of pKJK77 (or pKJK78 for the *sid71* allele). The resulting plasmid was called pKJK79 (or pKJK80 for the *sid71* allele). The pKJK79 (or pKJK80) insert is consisted of nearly half of the P4 genome and contains the *cos* region of P2 instead of the *cos* region of P4. The insert was taken by *Sal*I/*Mlu*I double digestion of pKJK79 (or pKJK80) and ligated with the *Sal*I/*Mlu*I double digested pKJK100 to make up the remaining portion of the P4 genome (see Fig. 2 for the construction procedure). The competent cells of the P2 lysogenic strain, *E. coli* C295, was transformed with the ligation mixture and poured onto an LB-agar plate with a fresh overnight culture of *E. coli* C295. The plaques appearing on that plate after overnight incubation were chosen as transfectant P4 derivatives containing the *cos* region of P2. Only the correctly ligated P4 portions made up the P4 genome having the *cos* region of P2 and multiplied as a P4 phage in the

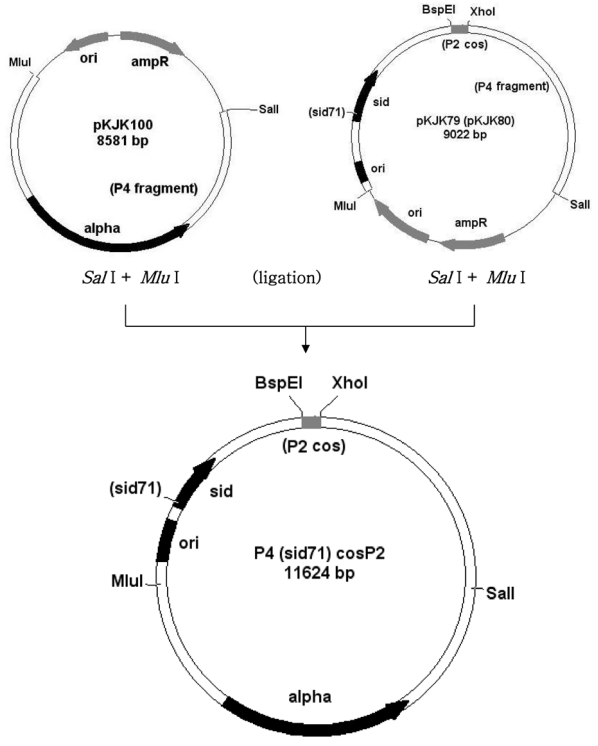

Fig. 2. Construction of P2 *sid71 cos*P2. The restriction enzyme cutting sites used in this construction are shown. Some genes, the exchanged P2 *cos* region, and *ori* of P4 are also shown. The thick white box indicates the P4 DNA portion and the thin line indicates the plasmid DNA portion.

Table 1. Burst size of P4 and derivatives

Phage	Burst size with C1a lysogenic for[a]	
	P2 *sir*+	P2 *sir2*
P4	121.5 ± 5.8	0.8 ± 0.1
P4 *cos*P2	89.4 ± 6.2	0.8 ± 0.1
P4 *sid71*	36.9 ± 9.6	4.4 ± 1.0
P4 *sid71 cos*P2	46.2 ± 10.9	51.7 ± 8.8

[a] All burst size data is the Mean±SD of more than three independent experiments

presence of helper P2 prophage in the *E. coli* C295 strain and formed a plaque. The concentrated phage stocks were made from those transfectants, according to Kim and Song (2006). The substitution of the P2 *cos* region for the P4 *co*s region and the presence of the *sid71* allele of the transfectant phages were identified by DNA sequencing. Finally, the *cos* region of P2 containing P4 derivative phages were called P4 *cos*P2 and P4 *sid71 cos*P2 for the one having a *sid71* allele.

At first, the burst sizes of P2 *cos* containing P4 derivatives, P4 *cos*P2 and P4 *sid71 cos*P2, were determined with wild type P2 lysogen (C295) and P2 *sir2* lysogen (C2142) hosts. To determine the burst size, one-step growth experiments were done according to the method previous used by Kim and Song (2006). All strains used in these experiments were derived from C1a, a type strain of *E. coli* C (Sasaki and Bertani, 1965). As shown in Table 1, the burst size for P4 *cos*P2 with wild type P2 lysogen was smaller than that for the P4 with wild type P2 lysogen. With P2 *sir2* lysogen, the burst size for P4 was the same as that for P4 *cos*P2. In the case of P4 *sid71 cos*P2, the burst size with P2 *sir2* lysogen was higher than that with the wild type P2 lysogen. Comparing the burst sizes for P4 *sid71* (4.4) and P4 *sid71 cos*P2 (51.7) with P2 *sir2* lysogen, there was more than ten times the increment in burst size for P4 *sid71 cos*P2. For P4 *sid71* and P4 *sid71 cos*P2, the ratio of burst size with P2 *sir2* lysogen to that with P2 lysogen was determined to observe the P2 *sir*-associated helper efficiency ratio (Table 2). The ratio of P4 *sid71 cos*P2 (1.12) showed that the substitution of the *cos* region of P2 for the *cos* region of P4 in P4 *sid71 cos*P2 overcame P2 *sir*-associated helper inefficiency.

It has been known that the prohead plays an important role in *cos*-cleavage and the packaging reaction in the P2-P4 bacteriophage system (Pruss *et al*., 1975). As the *sir* mutations reside on the *N* gene, there would be some difference between the prohead made of wild type *N* gene product (gpN) and the prohead made of mutant *N* gene product (gpN_{sir}) (Six *et al*., 1991). It might be possible that the prohead made of mutant *N* gene product (gpN_{sir}) prefers the *cos* region of P2 in *cos*-cleavage and packaging. A relatively high burst size and the P2 *sir*-associated helper efficiency ratio of P4 *sid71 cos*P2 compared to those of P4 *sid71* shown in this study support the notion that the *cos* region of P2 in P4 *sid71 cos*P2 may interact more efficiently with the prohead made of mutant *N* gene product (gpN_{sir}) in *cos*-cleavage and packaging. Therefore, the *cos* region of P2 is one of the important factors for P4 genomes packaged into the P2-size head in overcoming P2 *sir*-associated helper inefficiency.

Table 2. The ratio of burst size (B.S.) with P2 *sir2* to B.S. with P2

Phage	B.S. with P2 *sir2* lysogen/ B.S. with P2 lysogen
P4 *sid71*	0.12
P4 *sid71 cos*P2	1.12

The packaging of P2 *sid71 cos*P2 was examined using the CsCl buoyant equilibrium density gradient experiment, as described by Nilssen *et al.* (1996). Portions of phage stocks were mixed with CsCl solution and the average density adjusted to 1.38 g/ml. To obtain the density gradient, the mixtures were 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 tubes were punctured at the bottom and 28 fractions (400 μl for each fraction) 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. Each fraction was titrated for P4 PFUs (plaque forming units) using C353 as an indicator. The density profile was obtained by plotting the PFUs of each fraction against its density.

Figure 3 shows the profiles for P4 *sid71* and P4 *sid71 cos*P2. In the profile of the P4 *sid71* stock prepared with wild type P2 lysogen (Fig. 3A), two peaks appeared at the densities corresponding to the P2-size head packaged with three copies of the P4 genome and to the P2-size head packaged with two copies of the P4 genome, respectively. This profile is identical to that obtained in a previous study by Song and Kim (2006). However, in the case of the P4 *sid71 cos*P2 stock prepared with wild type P2 lysogen (Fig. 3B), only one peak appeared at the density corresponding to the P2-size head packaged with three copies of the P4 genome. This difference in density profile may reflects the difference in *cos*-cleavage and packaging between P4 *sid71* and P4 *sid71 cos*P2.

The phage stocks of P4 *sid71* and P4 *sid71 cos*P2 prepared with P2 *sir2* lysogen (C2142) as a host were examined using the CsCl buoyant equilibrium density gradient; their density profiles are shown in Figs. 3C and 3D. In the case of P4 *sid71* (Fig. 3C), only one peak appeared at the density corresponding to the P2-size head packaged with two copies of the P4 genome. The profile of the P4 *sid71* stock with the P2 *sir2* lysogen host was different from that of the P4 *sid71* stock with the P2 wild type lysogen host (compare Fig. 3A with Fig. 3C). The profile of the P4 *sid71 cos*P2 stock with the P2 *sir2*

lysogen host (Fig. 3D) showed the same pattern as that obtained with the P4 *sid71 cos*P2 stock with the P2 wild type lysogen host (Fig. 3B). In both profiles, only one peak appeared at the density corresponding to the P2-size head packaged with three copies of the P4 genome.

The difference in the packaging status of P4 *sid71* and P4 *sid71 cos*P2 with P2 *sir2* lysogen host may explain why P4 *sid71 cos*P2 overcomes P2 *sir*-associated helper inefficiency. The most plausible hypothesis is as follows: The *cos* region of P2 in P4 *sid71 cos*P2 is a more preferred substrate for *cos*-cleavage and packaging with the $P2_{sir}$-size prohead-containing complex than the *cos*-region of P4 in P4 *sid71*. Therefore, the genome of P4 *sid71 cos*P2 could be *cos*-cleaved and packaged to the same extent with the P2 wild type or P2 *sir* lysogens. Consequently, it appears that P2 *sir*-associated helper inefficiency is overcome by P4 *sid71 cos*P2.

In conclusion, the *cos* region of P2 containing the P4 derivative phage, P4 *sid71 cos*P2, was constructed through several DNA manipulation steps. Burst size determination and the CsCl buoyant equilibrium density gradient experiment with P4 *sid71 cos*P2 showed that the *cos* region of P2 in P4 *sid71 cos*P2 could be one of the important factors in overcoming P2 *sir*-associated helper inefficiency for a P4 genome packaged into a P2-size head.

적 요

박테리오파지 P2 *sir* 변이체는 그것의 위성파지인 박테리오파지 P4를 위해 비효율적인 도움파지로 알려졌다. 이러한 현상을 “P2 *sir*-관련 도움파지 비효율성”이라고 부르고 있으며, P2와 P4의 *cos* 지역에서의 DNA 염기배열 순서 차이가 이러한 현상을 나타나게 한다고 생각되었다. 이를 검증하기 위해, 여러 단계의 *in vitro* DNA 조작을 거쳐 P2의 *cos* 지역을 함유하는 유도체 P4인 P4 *sid71 cos*P2를 조성하였다. 일단계 생장 실험을 통해 그것의 후손 방출량을 결정하였다. 그 결과는, P4 *sid71 cos*P2에서 P4의 *cos* 지역을 P2의 *cos* 지역으로 대체한 것이 “P2 *sir*-관련 도움파지 비효율성”을 극복한 것으로 나타났다. P2 야생형과 P2 *sir* 돌연변이형을 도움파지로 삼아 준비한 P4 *sid71 cos*P2 파지 농축액들을 CsCl 부양 균등밀도 편차실험으로 분석하였다. 그 결과, 양 경우 모두 3개의 P4 유전체를 가진 파지 입자가 우세한 것으로 나타났다.

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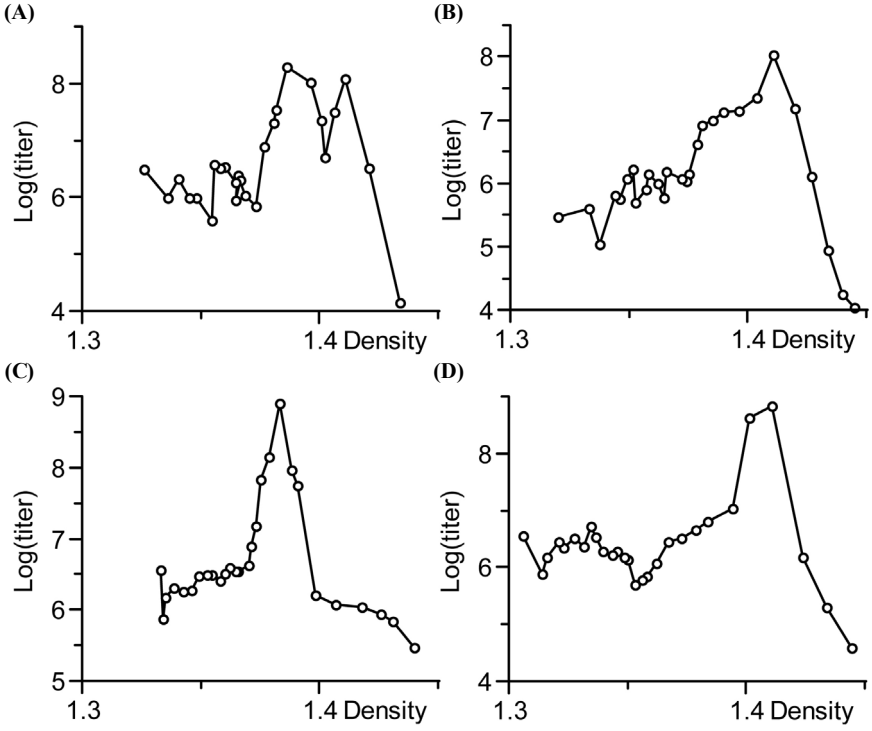

Fig. 3. CsCl buoyant equilibrium density gradient profiles of P4 *sid71* stock prepared with the w.t. P2 lysogen host (A); P4 *sid71 cos*P2 stock prepared with the w.t. P2 lysogen host (B); P4 *sid71* stock prepared with the P2 *sir2* lysogen host (C); and P4 *sid71 cos*P2 stock prepared with the P2 *sir2* lysogen host (D). The ordinates of these 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.

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