IMPROVED PHAGE VECTOR SYSTEM IN STREPTOMYCES

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SUMMARY: A phage vector system was established by use of R4 phage in Streptomyces. Increased transfection frequency, introduction of a single BamHI site as a possible cloning site, and simple and rapid methods for isolation of phage DNA were achieved for the establishment.

INTRODUCTION

Several workers, using plasmid vectors, succeeded in cloning genes in Streptomyces (Bibb, Schottel and Cohen, 1980; Thompson, Ward and Hopwood, 1980, 1982), while introduction of convenient phage vector system to this organism is much retarded. The phage vectors have some advantages; plaque hybridization can be used and the copy number of phages can be controlled by maintaining the prophage state or by inducing phage particles.

We have characterized temperate actinophage Bα from Streptomyces lavendulae (Nakano, Ishihara and Ogawara, 1981) and isolated deletion mutants (Ishihara, Nakano and Ogawara, 1982) with increased capacity for introducing larger foreign DNA fragments. Phage Bα then turned out to be identical with phage R4 isolated from soil by Chater and Carter (1979).

Isolation of phage DNA is time-consuming and troublesome compared with that of plasmid DNA. This paper describes improved methods for isolation of phage DNA, heightened transfection frequency, and facilitated introduction of foreign DNA by introduction of the BamHI site and of tyrosinase gene as a selection marker.

MATERIALS AND METHODS

Media: SAS and NAS media respectively denote soft agar (SA) and nutrient agar (NA) (Nakano et al., 1981) both supplemented with 0.3 M sucrose, 10 mM MgCl₂, 10 mM Ca (CO₃)₂ and 0.5% glucose.

Strains, phages and plasmid: S. lavendulae strain S55-B1 and S985 were gifted by Dr. A. Seino. Strain S985 was used as the host for transfection. Phage
R4 and a deletion mutant, A10, having 2.4 megadalton deletion in DNA, were described elsewhere (Ishihara et al., 1982, Nakano et al., 1981). Plasmid pIJ702, carrying the genes for tyrosinase and thiostrepton resistance (personal communication), was gifted by Prof. D. A. Hopwood.

**Construction of phage vector L24:** A10 DNA was prepared by the large scale method (Ishihara et al., 1982). A10 DNA (40 µg) was digested with PvuII and ligated with BamHI linker (2.5 µg) (Takara Shuzo, Co.). The ligation mixture, after redigestion with PvuII to eliminate A10 DNA, was transfected into S985 protoplasts.

**Transfection:** Protoplasts were obtained from mycelia of strain S985 at the middle of the log growth phase in Tryptic Soy Broth (Difco Labs) supplemented with 1% glycine. Transfection was done by a modified method of Isogai et al. (1980) except for omitting two washing steps after treatment with lysozyme and polyethylene glycol. The omission did not affect the transfection frequency.

**Isolation of tyrosinase gene from pIJ702:** Plasmid pIJ702, after digestion with BclI and BamHI, electrophoresed in 4% polyacrylamide gel (Maniatis, Jeffrey and van de Sande, 1975). Corresponding to the fragment containing tyrosinase gene (Katz et al., personal communication), the band of the largest fragment was cut out from the gel and extracted by the electroelution method (Smith, 1980). The DNA extracted was inserted into the BamHI site of L24.

**RESULTS**

The Effects of the Age and Number of Protoplasts on the Transfection Frequency

We have reported that the transfection efficiency was about $2 \times 10^4$ per µg of Bα DNA. Using S985 cells at various ages, we tried to increase the efficiency. The S985 spores (10⁶ colony-forming units) were allowed to grow in Tryptic Soy Broth and the grown mycelia were harvested in 23 (early log phase), 28 (late middle log phase) and 42 hr (early stationary phase). Various amounts of protoplasts prepared from each were transfected with 0.1 µg of R4 DNA. The maximal transfection frequency was obtained with those harvested in 28 hr (Fig. 1). The relationship between the protoplast concentration and the transfection frequency is shown also in Fig. 1. One OD₆₆₀ unit corresponded to about $2 \times 10^6$ colony-forming units. At the maximal frequency, 10⁶ transfectants per µg of DNA or approximately one transfectant per protoplast were obtained. Concentrations of protoplasts higher than $2 \times 10^6$ decreased the transfection efficiency.

Rapid and Simple Method for Preparation of R4 DNA and Its Derivatives

We established two rapid and simple methods for isolation of phage DNA: one was a small-scale method yielding relatively pure DNA for restriction enzyme analysis (Table I) and the other a mini-scale method yielding relatively crude but enough DNA to detect recombinant phage DNA by examining the distribution of fragments formed by a restriction enzyme (Table II). By the
Fig. 1. Effects of age and concentration of protoplast on transfection frequency. Concentration of protoplast was expressed by absorbance at 660 nm. S. lavendulae S985 spores were grown in Tryptic Soy Broth and the grown mycelia were harvested at 23, 28 and 42 hr for preparing protoplasts.

(a) Transfection frequency was determined from the number of plaques per μg DNA.
(b) Transfection frequency was determined from the number of plaques per protoplast.
•; 23 hr, ○; 28 hr, □; 42 hr.

small-scale and mini-scale methods, 1 μg or more and 0.1 μg or more of DNA were obtained in 2 days and within several hours, respectively.

Construction of Phage Vectors

The deletion mutant, A10, has a single restriction site for each of PvuII and PstI. As Streptomyces chromosomal DNA is relatively resistant to these enzymes and the ligation between the blunt ends formed by PvuII cleavage is less efficient than that between the cohesive ends, the BamHI restriction site was introduced into A10 DNA (see Materials and Methods). From 10 plaques obtained by transfecting S985 protoplasts with the ligation mixture, DNA was prepared by the procedures given in Table II. Four clones, L24, L26, L29 and L30, were found to have the BamHI site. Figure 2 shows that A10 DNA had no site for cleavage by BamHI and that L24 DNA had a single BamHI site.
TABLE I

Small-scale preparation of phage DNA

1. Plate phages with 5985 spores by the double layer method and incubate overnight the plates.
2. Scrape top layers into 2 volumes of TBT buffer (0.1 M Tris-HCl, pH 7.2, containing 0.1 M NaCl 0.01 M MgCl₂ and 0.01 M Ca (CO₃)₂).
3. Sterilize phage solution by membrane filtration (phage stock solution, that can be stored at 4°C).
4. One ml of phage stock solution was mixed with 9 ml of SA supplemented with 10⁷–10⁸ colony-forming units of spore suspension.
5. Plate and incubate the plates for 20 hr at 30°C.
6. Scrape all of agar into 10 ml of TBT buffer.
7. Centrifuge (7,000 ×g, 10 min at 4°C) to remove agar.
8. Centrifuge (40,000 ×g, 90 min at 2°C) to concentrate phage.
9. Resuspend in 400 μl of TBT buffer containing DNase (1 μg/ml) and incubate it at 37°C for 1 hr.
10. Add 100 μl of 0.5 M Tris-HCl, pH 9.6, 0.25 M EDTA, 25% SDS.
11. Heat at 70°C for 30 min.
12. Add 125 μl of 8 M potassium acetate at 4°C.
13. Centrifuge (30,000 ×g, 20 min at 2°C) to remove SDS.
14. Add 2 volumes of cold ethanol and store at -70°C for 10 min.
15. Resuspend in 300 μl of 0.01 M Tris-HCl, pH 7.6, containing 0.01 M NaCl and 0.001 M EDTA.

TABLE II

Mini-scale preparation of phage DNA

1. Mix 0.1 ml of phage extract, 0.2 ml of double-strength nutrient broth and 0.1 ml of S985 spores.
2. Incubate for 18 hr at 30°C with shaking.
3. Centrifuge to remove mycelia.
4. Add 150 μl of 1.2 M Tris-HCl, pH 9.6, containing 0.2 M EDTA and 1% SDS.
5. Heat for 30 min at 70°C.
6. Add 150 μl of chilled 8 M potassium acetate.
7. Centrifuge (30,000 ×g, 20 min at 2°C) to remove SDS.
8. Add 2 volumes of cold ethanol and store at -70°C for 10 min.
9. Ethanol precipitation to desalt and concentrate DNA.
10. Resuspend in 300 μl of 0.01 M Tris-HCl, pH 7.6, containing 0.01 M NaCl, 0.001 M EDTA and 1 M LiCl.
11. Extract with chloroform.
12. Precipitate with ethanol.
13. Extract with chloroform.

Since the BamHI site is dispensable for phage function, various DNA fragments digested with BamHI, BclI, BglII and MboI can be inserted into the BamHI site.
Use of L24 as a Cloning Vector

Chromosomal DNA of S. lavendulae S55-B1 was cleaved with MboI and the DNA fraction corresponding to about 4 megadaltons was electroeluted from the gel and ligated with BamHI-digested L24. After redigestion with BamHI to remove L24 DNA, the ligation-mixture was transfected into S985. From each
Fig. 4.
plaque obtained, phage DNA was purified by the method given in Table II and recombinant phage DNA was screened by BclII cleavage. As described previously (Ishihara et al., 1982), R4 had more than 12 sites susceptible to cleavage by BclII and the second largest BclII fragment contained the BamHI site which was used for the introduction of foreign DNA, thus the presence and the size of the insertion fragment were easily determined by the pattern of DNA cleavage with BclII. Figure 3 shows that the second largest fragment originating from the recombinant phage carrying a chromosomal DNA segment was larger than that from L24 by 1.2 to 1.3 megadaltons as indicated by arrows.

**Introduction of Tyrosinase Gene into L24**

To construct L24 phage with a selection marker, we tried to introduce tyrosinase gene from pIJ702 plasmid into L24 DNA. Since tyrosinase is involved in melanin production, transformants with pIJ702 plasmid can be easily detected by a black, diffusible pigment around the colony. This melanin production is useful as a selection marker if this gene is expressed in such a phage vector as L24. A BclII fragment of one megadalton DNA encoding tyrosinase was subcloned to the BamHI site of L24 as described in Materials and Methods. Cleavage of L24 with BglII forms 13 fragments and the largest fragment A contains a BamHI site (Fig. 4A and C). As the BclII fragment of tyrosinase gene has a single BglII site, insertion of the tyrosinase gene into L24 DNA can be discerned by the appearance of an additional BglII fragment X (Fig. 4A, B and C). After purification by the method described in Table II and screening phage DNA with BglII digestion, 7 among 33 phage DNAs were found to have DNA containing tyrosinase gene. The mode of insertion of tyrosinase gene was determined by using two recombinant phages, I406 and I409. The tyrosinase gene has a single SstII site; the distance between the BglII and SstII sites is 0.16 megadaltons; L24 has no site for cleavage by SstII. Therefore, the direction of insertion was determined by digestion with both BglII and SstII. The BglII fragment X of I406 was not changed upon additional digestion with SstII; whereas the fragment X of I409 was shortened upon SstII digestion from 4.9 to 4.7 megadaltons. These results were consistent with the direction determined as above.

The two recombinant phages were allowed to infect S985, which was plated on NA or R2 medium (Okanishi, Suzuki and Umezawa, 1974) supplemented with yeast extract (0.5%), methionine (100 µg/ml) and tyrosine (400 µg/ml). No melanin production was detected in the plaques, but S985 lysogens of the phages produced melanin.

**DISCUSSION**

It is difficult to use phages as the cloning vectors since isolation of phage DNA is time-consuming. Such difficulty was overcome by this investigation. In the mini-scale method, the dialysis step was omitted, which enabled us to obtain...
phage DNA in only several hours.

The single *Bam*HI site introduced to R4 phage proved useful for gene cloning. In phage λ, 80 to 105% of DNA of the normal size is being packaged. As the molecular weight of R4 phage DNA is 34 megadaltons, maximal packaging in L24 will theoretically be about 4 megadaltons, since it has deletion of 2.4 megadaltons.

**REFERENCES**


