Construction of Phage Vectors in *Streptomyces*: Introduction of the Thiostreptone Resistant (*tsr*) Gene into R4 Phage

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We have constructed phage cloning vectors from an actinophage, R4. A deletion derivative (R4 Δ 22B) which had a *Bam*HI linker inserted at the unique *Pvu*II site was used to clone the thiostreptone resistant (*tsr*) gene derived from plasmid vector pIJ365. The *tsr* derivative obtained, R4 Δ 22B-*tsr*1, was shown to have the same level of thiostreptone resistance in lysogenized cells as that of pIJ365-carrying cells. Under the optimal conditions, R4 Δ 22B-*tsr*1 phage was lysogenized at a frequency of 5×10^{-2} per infected phage. The usefulness of R4 phage derivatives for gene cloning is discussed.

Streptomyces strains are notable as producers of medically important antibiotics. We have been working on construction of phage vector systems in *Streptomyces* strains. An actinophage, R4, and its deletion mutants are useful as cloning vectors.^{1,2)} Also we reported construction of a restriction map of the R4 phage genome.³⁾ Although R4 phage and its deletion derivatives are quite useful for gene cloning in *Streptomyces*, it is desirable to add a selective marker to the R4 cloning vectors. In the case of *coli*-phage lambda, a lactose-utilizing gene (*lac*) has been used for this purpose.⁴⁾ Since R4 is a temperate phage, we can introduce antibiotic resistant markers into it to select the lysogens.

In this report we describe construction of a R4 phage derivative having an antibiotic resistant gene. Expression of the resistant gene in the phage and transduction of the phage are discussed.

MATERIALS AND METHODS

a) *Bacterial and phage strains.* *Streptomyces lividans* 66 and *S. parvulus* 2297 (*cys*) were described in a previous

paper.⁵⁾ R4 Δ 2 is a deletion mutant of R4 phage.¹⁾ *S. lividans* TK123 carrying pIJ365 was kindly provided by D. A. Hopwood.

b) *Media.* NB medium was used for germination of spores, phage propagation, phage titration and the cross-streak test. NB medium consisted of 0.8% Nutrient broth (Difco), 5 mM $\text{Ca}(\text{NO}_3)_2$ and 0.5% glucose. NB agar and NB soft agar contained 1.5% and 0.7% Bacto-agar (Difco), respectively. YS agar plates were used for spore formation and phage lysogenization. YS agar medium consisted of 0.2% Yeast extract (Difco), 1% soluble starch and 1.5% Bacto-agar (Difco) (pH 7.0). Minimal agar medium was the same as described in a previous paper.⁵⁾ Thiopeptin was kindly provided by Fujisawa Pharmaceutical Co. and used for detection of thiostreptone resistance.

c) *Purification of DNA, cleavage analysis and transfection.* Plasmid pIJ365 was prepared by the method of Ish-Horowicz and Burke.⁸⁾ For restriction analysis, plasmid DNA was purified by CsCl-ethidium bromide and sucrose density gradient centrifugation. Cleavage analysis and transfection were performed as described previously.^{3,5)}

d) *Insertion of BamHI linker into the R4 phage genome.* R4 phage DNA digested with *Pvu*II endonuclease was mixed with *Bam*HI linker (Genex Co., Ltd.) at a molecular ratio of 1:50 ligated with T4 DNA ligase. The ligated DNA was redigested with *Pvu*II endonuclease to

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eliminate self-ligated DNA molecules. Transfection was performed using protoplasts of *S. lividans* or *S. parvulus* as the recipients.⁵⁾

e) *Induction of R4 lysogens.* About 5×10^8 colony forming units (c.f.u.) of R4 lysogenized spores were inoculated into 5 ml of NB medium. After 6~8 hr cultivation at 30°C with shaking, mitomycin C (Kyowa Hakko, Co.) was added to the medium to a final concentration of 10 µg/ml. Then the cultivation was continued for a further 3 hr and cells were removed by centrifugation at 3,000 rpm for 10 min. The phage lysate was filtered through a 0.22 µm pore-size nitrocellulose filter.

f) *Minimal inhibitory concentration (MIC) of thiopeptin.* A homogenized spore suspension of about 5×10^7 c.f.u. per ml was prepared and 10 µl of this suspension was used for spot-tests on YS agar plates containing various amounts of thiopeptin. After two days incubation at 27°C, the growth response of the spores was examined.

RESULTS AND DISCUSSION

a) Isolation of deletion mutants

Deletion mutants of R4 phage were isolated from R4Δ2 by a pyrophosphate enrichment method.¹⁾ A phage suspension was treated with 25 mM pyrophosphate for 10 min at 30°C and surviving phages (About 10^{-2}) were propagated without pyrophosphate. By repeating the pyrophosphate enrichment procedure seven times, the pyrophosphate resistance of the final phage lysate increased to 1.2×10^{-1} . Another cycle of pyrophosphate treatment was performed and then the phage lysate was subjected to single-plaque isolation. Twelve

clones were obtained and further examined. As a result we isolated two clones which have new deletions besides the Δ2 deletion. The two deletions were named Δ21 and Δ22. Properties of the deletion mutants are summarized in Table I.

The R4Δ22 mutant can form plaques on *S. parvulus* but not on *S. lividans* although the phage mutant lysogenizes normally to both strains. However, the R4Δ21 mutant cannot lysogenize to the same strains. So the region of the Δ21 deletion is essential for the lysogenization.

b) Introduction of the *Bam*HI site into the R4 genome (Table II)

We have previously shown that R4 phage DNA has a unique *Pvu*II site in a dispensable region, which can be used for a cloning site.²⁾ The *Pvu*II site is quite useful for inserting DNA fragments with blunt ends, but not adequate for cloning other restriction fragments. R4 phage DNA has no *Bam*HI site. So *Bam*HI linker was introduced into the *Pvu*II site of R4Δ2, R4Δ21 and R4Δ22 phage DNAs. The resultant *Bam*HI site-bearing derivatives of these deletion mutants were named R4Δ2B, R4Δ21B and R4Δ22B, respectively. The presence of the *Bam*HI site in R4Δ22B is shown in Fig. 2 (lane 2) and Fig. 3 (lanes 2 and 5). The presence of *Bam*HI sites in R4Δ2B and R4Δ21B was also confirmed by cleavage analysis (data not shown). The deletion sizes of the R4Δ2B, R4Δ21B and R4Δ22B mutants are 2.7 kb, 4.2 kb and 5.2 kb, respectively. So we can clone at least 5.2 kb DNA fragments with cohesive ends identical to those of the *Bam*HI sites.

TABLE I. PYROPHOSPHATE RESISTANCE OF R4 PHAGE DERIVATIVES

Strain	Deletion (kb)	Surviving fraction ^a
R4 wild	0	1.3×10^{-5}
R4Δ2	2.7	2.7×10^{-2}
R4Δ21	2.7+1.5	1.0×10^{-1}
R4Δ22	2.7+2.5	2.0×10^{-1}
R4Δ22B- <i>tsr</i> 1	3.3	6.9×10^{-2}

^a Phage strains were treated with 50 mM sodium pyrophosphate in 50 mM Tris-HCl (7.5) at 37°C for 10 min. Surviving fractions were calculated from the titer before and after the treatment. R4Δ21 and R4Δ22 have new 1.5 kb and 2.5 kb deletions, respectively, besides the 2.7 kb deletion of R4Δ2.

TABLE II. EFFECT OF DELETIONS ON PLATING EFFICIENCY

Phage strain	E.O.P. ^a
R4 wild	0.23
R4Δ21	0.14
R4Δ22	$< 1.0 \times 10^{-8}$

^a Efficiency of plating (E.O.P.) signifies the ratio of E.O.P. on *S. lividans* to that on *S. parvulus*.

c) Introduction of the *tsr* gene into the R4 genome

To construct R4 phage derivatives having an antibiotic resistant gene, the thiostreptone resistant gene (*tsr*) of plasmid pIJ365 was introduced into the *Bam*HI site of R4Δ22B phage DNA. The construction is schematically illustrated in Fig. 1. R4Δ22B and pIJ365 DNAs which had been cleaved with *Bam*HI endonuclease, were mixed at a molecular ratio of 1:5 and ligated with T4 DNA ligase. The ligated DNA was transfected to *S. parvulus* and about 300 phage clones were obtained. These clones were mixed and lysogenized to *S. parvulus*. After sporulation of the putative lysogens, spores were replica-plated on YS agar plates containing 50 μg/ml of thiopeptin. Seven thiopeptin resistant clones were obtained. These clones were confirmed to be R4 lysogens as phages were induced by mitomycin C treatment. After repeated single plaque isolation, all of the phage clones were shown to give thiopeptin resistance by their lysogenization to *S. parvulus*. One clone, named R4Δ22B-*tsr*1, was used for further characterization.

Since the R4Δ22B-*tsr*1 phage shows a thiopeptin resistant phenotype upon lysogenization, the phage should have the *tsr* gene. To confirm the presence of the *tsr* gene in R4Δ22B-*tsr*1 phage DNA, cleavage analysis of the phage DNA was performed. The results of agarose gel electrophoresis are shown in Figs. 2 and 3. As shown in lanes 2 and 3 of Fig. 2, *Bam*HI digests of R4Δ22B and R4Δ22B-*tsr*1 DNAs gave apparently identical bands. A predicted 4.5 kb *Bam*HI fragment derived from pIJ365 was not observed in the *Bam*HI digest of R4Δ22B-*tsr*1 DNA. This result suggests that the *Bam*HI fragment containing the *tsr* gene was inserted into the *Bam*HI site of R4Δ22B and that one of the *Bam*HI sites was deleted. This was confirmed by double digestion experiments. When the R4Δ22B-*tsr*1 DNA was digested with *Bam*HI and *Pst*I, 0.72 and 5.4 kb fragments were observed. The smaller fragment was identical to that of pIJ365 cleaved with *Bam*HI and *Pst*I, and the larger

FIG. 1. Construction of the R4Δ22B Derivative Containing the *tsr* Gene.

*Bam*HI-cleaved R4Δ22B phage DNA was ligated with *Bam*HI-cleaved pIJ365 DNA and transfected to *S. parvulus*. Resultant transfectants were lysogenized to *S. parvulus* and thiopeptin resistant clones were obtained. One of such clones was induced and the phage obtained was named R4Δ22B-*tsr*1. A *Bam*HI DNA fragment derived from pIJ365 containing the *tsr* gene is shown as a heavy line and an arrow. The lower part shows the restriction map of R4Δ22B-*tsr*1 phage. An open box shows a deleted region and numbers below the restriction map indicate distances between restriction sites (kb). Restriction sites and the number in parentheses signify putative restriction sites and the size, respectively.

FIG. 2. Agarose Gel Analysis of R4 Derivatives.

R4Δ22 DNA (lane 1), R4Δ22B DNA (lane 2) and R4Δ22B-*tsr*1 DNA (lane 3) were digested with *Bam*HI restriction endonuclease and analyzed on agarose gel. Lane 4 shows lambda DNA digested with *Hind*III.

FIG. 3. Cleavage Analysis of R4Δ22B-*tsr*1 Phage DNA.

DNA was digested with restriction enzymes and the fragments were analyzed by 0.8% agarose gel electrophoresis. Lane 1, *Hind*III-digested lambda DNA (reference). Lane 2, *Bam*HI-*Pst*I-digested R4Δ22B DNA. Lane 3, *Bam*HI-*Pst*I-digested R4Δ22B-*tsr*1 DNA. Lane 4, *Bam*HI-*Pst*I-digested pIJ365 DNA. Lane 5, *Bam*HI-*Cla*I-digested R4Δ22B DNA. Lane 6, *Bam*HI-*Cla*I-digested R4Δ22B-*tsr*1 DNA. Lane 7, *Bam*HI-*Cla*I-digested pIJ365 DNA. Lane 8, *Bcl*I-digested R4Δ22B DNA. Lane 9, *Bcl*I-digested R4Δ22B-*tsr*1 DNA. Arrows in lanes 4 and 7 indicate the 0.72 and 1.92 kb fragments shown in Fig. 1.

TABLE III. EFFECT OF MEDIUM AND ANTIBIOTIC ADDITION TIME ON LYSOGENIZATION FREQUENCY

Time (hr) ^a	Lysogenization frequency (CFU/PFU) ^b	
	YS	MM
0	2.5×10^{-5}	2.5×10^{-5}
2	7.5×10^{-5}	1.0×10^{-3}
3	4.0×10^{-4}	3.4×10^{-3}
6	8.5×10^{-4}	7.5×10^{-3}
8	3.7×10^{-3}	ND ^c
14.5	1.5×10^{-2}	3.0×10^{-2}
17	1.8×10^{-2}	5.4×10^{-2}
20	—	6.0×10^{-2}

^a Germinated spores of *S. parvulus* were infected with R4Δ22B-*tsr*1 at m.o.i. = 0.1. After 20 min incubation at 27°C, infected cells were spread on YS or MM plates. At the indicated times, 3 ml of NB soft agar containing 50 μg/ml thiopeptin was overlaid.

^b Lysogenization frequency is shown as colony forming units (c.f.u.) per phage infected (p.f.u.).

^c Not determined.

fragment was identical to that of R4Δ22B cleaved with *Bam*HI and *Pst*I. Since the *Pst*I site of R4Δ22B DNA is located on the left-hand side of the *Bam*HI site and the 5.4 kb *Bam*HI-*Pst*I fragment of R4Δ22B-*tsr*1 DNA is intact, the left-hand side of the *Bam*HI site

FIG. 4. Dose Response of Transductants to m.o.i. in R4Δ22N-*tsr*1 Phage Infection.

Germinated spores of *S. parvulus* were infected with R4Δ22B-*tsr*1 phage at increasing m.o.i. and thiopeptin resistant clones were counted. Other conditions were the same as described in the legend to Table III.

should be intact (see Fig. 1 and lanes 2~4 of Fig. 3). Also *Bam*HI-*Cla*I digested R4Δ22B-*tsr*1 DNA gave a 1.92 kb fragment identical to that of pIJ365 cleaved with *Bam*HI and *Cla*I (lanes 6 and 7). R4Δ22B DNA has no *Cla*I site (lane 5). If we compare *Bcl*I-digested fragments of R4Δ22B and R4Δ22B-*tsr*1 DNAs, a 7.6 kb fragment of R4Δ22B DNA increases in size to 9.5 kb in R4Δ22B-*tsr*1 DNA (lanes 8 and 9). Therefore we concluded that the *Bam*HI fragment containing the *tsr* gene was inserted

TABLE IV. CHARACTERISTICS OF R4 PHAGE VECTORS

Strain	Deletion(kb)	Cloning site	Comments
R4Δ2	2.7	<i>PvuII</i>	
R4Δ21	4.2	<i>PvuII</i>	<i>cI</i> like
R4Δ22	5.2	<i>PvuII</i>	No growth on <i>S. lividans</i>
R4Δ2B	2.7	<i>BamHI</i>	
R4Δ21B	4.2	<i>BamHI</i>	Same as R4Δ21
R4Δ22B	5.2	<i>BamHI</i>	Same as R4Δ22
R4Δ22B- <i>tsr</i> 1	3.3	<i>BamHI</i> , <i>SmaI</i> , <i>PvuII</i> , <i>ClaI</i>	No growth on <i>S. lividans</i> , <i>tsr</i> marker, insertional inactivation (<i>ClaI</i>)

into the *BamHI* site of R4Δ22B and that the right-hand side of the region was deleted (Fig. 1). The deletion size was estimated to be about 1.9 kb. So the genome size of R4Δ22B-*tsr* 1 is about 3.3 kb smaller than that of wildtype R4. As shown in Fig. 1, we found one *PstI*, one *SmaI* DNA insertion. Also two *PvuII* sites can be used for gene cloning. Especially the latter three sites are in the *tsr* gene and useful for insertional inactivation.

R4Δ22B-*tsr* 1 phage DNA has three unique sites, *BamHI*, *SmaI*, and *ClaI*, available for DNA insertion. Also two *PvuII* sites can be used for gene cloning. Especially the latter three sites are in the *tsr* gene and useful for insertional inactivation.

d) *Optimal conditions for R4Δ22B-*tsr* 1 phage lysogenization*

R4Δ22B-*tsr* 1 phage shows thiopeptin resistance upon lysogenization. To determine the optimal conditions for lysogenization of R4 phage, R4Δ22B-*tsr* 1 phage was used. When *S. parvulus* 2297 was used as the recipient, poor media such as minimal medium were suitable for a high frequency of lysogenization. Maximum lysogenization was attained when the phage-infected spores were incubated for 16 hr at 27°C before thiopeptin addition. The lysogenization frequency was about 5×10^{-2} per infected phage (Table III).

Figure 4 shows that the number of thiopeptin resistant clones increases linearly with increasing M.O.I. At a high M.O.I., the frequency declined presumably due to the killing effect of infecting phages.

The lysogens were quite stable even in the absence of the drug. This will be quite impor-

tant for use of the phage vector as a low copy number vector.

e) *Minimal inhibitory concentration of thiopeptin for R4Δ22B-*tsr* 1 lysogens*

The minimal inhibitory concentration (MIC) of thiopeptin was examined using *S. parvulus* lysogenized with R4Δ22B-*tsr* 1 phage. The MIC was 3 μg/ml for R4Δ22B lysogen and 100 μg/ml for R4Δ22B-*tsr* 1 lysogen. The MIC value for R4Δ22B-*tsr* 1 lysogen is almost equivalent to that of *S. parvulus* carrying the pIJ365 plasmid. As the pIJ365 is a multi-copy type,⁶⁾ the thiopeptin resistant level in the R4Δ22B-*tsr* 1 lysogen seems to be quite high if the genome number of R4 phage in the lysogenic state is equal to that of the host chromosome. This is also the case in actino-phage φC31.⁷⁾

Biological and physical characterization of R4 phage derivatives described in this and previous papers¹⁻³⁾ are summarized in Table IV.

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