

Characterization of Recombinant Derivatives of pJY711 of Multicopy *Streptomyces* Plasmid

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Multicopy *Streptomyces* 플라스미드 pJY 711의 재조합 유도체의 특성

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ABSTRACT: The restriction cleavage map of multi-copy recombinant plasmid, pJY712 (8.1kb), carrying the thiostrepton resistance gene (*tsr*) was determined. pJY712 had a broad host range in *Streptomyces* and contained single *Bgl*II site for cloning purpose. The plasmid showed the phenomenon of lethal zygosis (Ltz^+). Transformation frequency of pJY712 was 5.0×10^4 transformants per μ g plasmid DNA (TFU) in *S. lividans*. Plasmid pJY713 was constructed by inserting the tyrosinase gene (*mel*) into the *Bcl*I site of pJY712. Recombinant plasmid pJY714 carrying the *mel* gene was constructed by *in vitro* deletion of a segment (1.9 kb *Bgl*II-*Bcl*I fragment) from pJY713.

KEY WORDS: *Streptomyces*, plasmid vector, broad host range

With recent developments in *Streptomyces* genetics, it becomes possible to clone genes and improving the strains (Chater *et al.*, 1982; Hopwood and Chater, 1982; Bibb *et al.*, 1983). An important development in *Streptomyces* genetics has been the isolation and characterization of *Streptomyces* plasmids as potential vectors. For the application of cloning of foreign genes in *Streptomyces*, in application of specific genes or expression of genes in different hosts, plasmids and derivatives of high copy number and broad host range have been reviewed. Several high copy number plasmid, such as pIJ101 from *Streptomyces lividans* ISP5434 (Kieser *et al.*, 1982), pUC6 from *S. spinosus* (manis *et al.*, 1982) and pJV1 from *S. phaeochromogenes* (Doull *et al.*, 1983; Bailey *et al.*, 1986) have been described and their derivatives also have been constructed as useful vectors. For similar reason, we have been studied for the development of new plasmid vector in *Streptomyces* which is small in size large in copy number useful for cloning and transformation purpose. In recent paper (Yu *et al.*, 1987), we reported the isolation

of a small (7.0 kb) and multi-copy (about 35 per chromosome) plasmid, pJY711, isolated from *Streptomyces* sp. and the construction of the derivative, pJY712, carrying the thiostrepton resistance gene (*tsr*).

In this communication, we report the characteristics of small and multi-copy derivatives of pJY711 (Yu *et al.*, 1987) compared with useful cloning vectors already in use.

MATERIALS AND METHODS

Bacterial strains and plasmids

The pJY711 and the recombinant plasmid pJY712 carrying the thiostrepton resistance gene (*tsr*) that had already been studied by the present authors (Yu *et al.*, 1987) was used throughout this study. *S. lividans* TK24 (Hopwood *et al.*, 1983) used the standard host and pIJ702 (Katz *et al.*, 1983) and pIJ41 (Thompson *et al.*, 1982b) were donated by professor D.A. Hopwood, John Innes Institute, Norwich, UK. Other *Streptomyces* strains used host range

Table 1. Transformation of pJY712 into various *Streptomyces*

Strains	Transformation by pJY712*
<i>S. albus</i> subsp. <i>albus</i> ATCC29795	Low **
<i>S. antibioticus</i> IFO12838	Low
<i>S. aureus</i> ATCC21428	—
<i>S. avermitilis</i> ATCC31267	Low
<i>S. cacaoi</i> subsp. <i>asoensis</i> IFO13813	—
<i>S. coelicolor</i> IAM1023	High
<i>S. flaveolus</i> IFO3715	High
<i>S. griseus</i> IFO12875	—
<i>S. griseus</i> subsp. <i>griseus</i> IFO13189	—
<i>S. hygrosopicus</i> ATCC 21705	—
<i>S. kasugaensis</i> ATCC15715	—
<i>S. lividans</i>	High
<i>S. mediocidicus</i> ATCC13278	—
<i>S. mitakaensis</i> ATCC15295	High
<i>S. tandeae</i> ATCC31160	—
<i>N. mediterranei</i> IFO13142	—

*; pJY712 DNA was isolated from *S. lividans*.

**; Low, lower than 10^3 transformants per ug plasmid DNA; High, higher than 10^3 transformants per ug plasmid DNA.

—; No transformant.

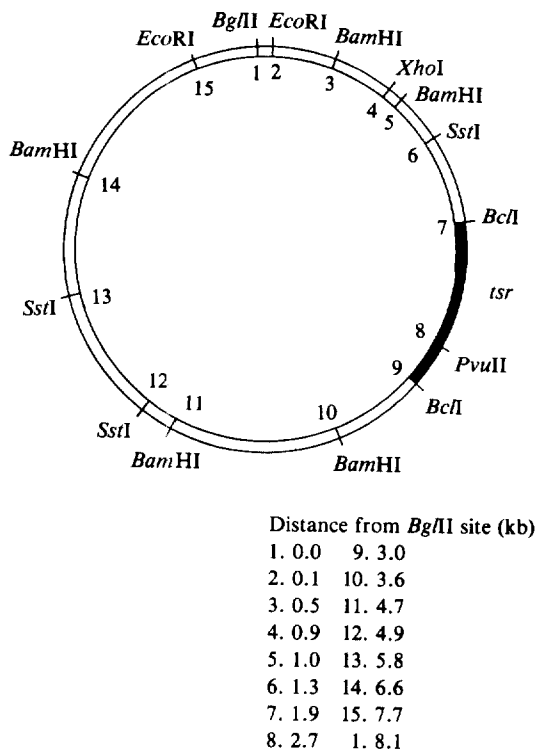
studies are listed on Table 1. *E. coli* HB101 (Bolivar *et al.*, 1977a) was used as host for pBR322 (Bolivar *et al.*, 1977b) and bifunctional derivatives.

Culture condition and transformation procedure

All *Streptomyces* for transformation experiments were grown in YEME medium containing 34% sucrose, 0.2% $MgCl_2 \cdot 6H_2O$ and 0.5% glycine. Protoplast formation and transformation of *S. lividans* were as in Thompson *et al.* (1982a). Direct selection for thiostrepton resistant transformants was made by overlaying regeneration medium (R2YE) with 2.5 ml soft Nutrient agar (Difco) containing 500 ug thiostrepton per ml after incubation for 20 hrs (Kieser *et al.*, 1982). Thiostrepton (kindly donated by Mr. S.J. Lucania of E.R. Squibb and Son, New Brunswick, NJ, USA) was used in liquid at 5 ug per ml and solid media at 50 ug per ml. *E. coli* HB101 was transformed as described by Nocard *et al.* (1978).

DNA manipulation

Plasmid isolation, restriction enzyme digestion, bacterial alkaline phosphatase treatment, agarose gel electrophoresis and ligation of DNA were essentially as Hopwood *et al.* (1985).

**Fig. 1.** Restriction map of pJY712.

No sites found for *Hind*III, *Xba*I, *Kpn*I and *Pst*I.

RESULT

Restriction endonuclease cleavage map of pJY712

The restriction cleavage map of pJY712 were determined from cleavage patterns obtained by successive digestion with various combination of restriction enzyme (Fig. 1). Comparison of the restriction patterns with those of plasmids previously (i.e., SCP2°, SLP1.2 (Bibb *et al.*, 1980), pIJ101 (Kieser *et al.*, 1982), pUC6 (Manis and Highlander, 1982), pSK1 and pSK2 (Toyama *et al.*, 1981), pFJ103 (Richardson *et al.*, 1982) and pJV1 (Doull *et al.*, 1983) demonstrated that the isolated plasmid from soil, pJY711, was novel.

Lethal zygosis and compatibility of pJY711 with other plasmids

The known plasmids of *Streptomyces* exhibit a property that have been called lethal zygosis (Ltz⁺) correlated with plasmid transfer (hopwood *et al.*, 1983). We could detect a lethal zygosis in regenerating *S. lividans* lawns after transformation with pJY711 (Yu *et al.*, 1987). The compatibility of pJY711 and pIJ702 (having the replicon of pIJ101; Kieser *et al.*, 1982) was investigated by transforma-

tion of *Streptomyces* sp. No. 71 (Yu et al., 1987) carrying pJY711 with pIJ702. All thiostrepton-resistant colonies tested had both pJY711 and pIJ702, but an apparent copy number of pIJ702 on agarose gel was higher than pJY711. Also pJY711 was compatible with pIJ41 containing the essential region of SLP1.2 (Thompson et al., 1982b). Therefore, the result showed that two families of multi-copy plasmids compatible.

Host range of pJY712

The host range of pJY712 was determined by protoplast transformation, using a *Streptomyces* series different with that used by Kieser et al. (1982). Selection for thiostrepton resistance was made by overlaying the regenerating protoplasts after 20 hrs with soft agar containing thiostrepton. The results are shown in Table 1. The confirmation of transformation was made in each case by isolation of plasmid DNA, except in the case of *Nocardia mediterranei* IFO13142 where partially thiostrepton-resistant colonies were obtained on transformation with pJY712 but no plasmid DNA could be detected by DNA isolation. *S. kasugaensis* ATCC 15715, *S. aureus* ATCC21658, *S. cacaoi* subsp. *asoensis* IFO13813, *S. hygrosopicus* ATCC 21705, *S. griseus* IFO12875, *S. griseus* subsp. *griseus* IFO13189, *S. mediodicidicus* ATCC 13278 and *S. tandeae* ATCC31160 were not transformed by pJY712. The low frequencies observed and failure of transformations for several species could be explained either by the non-optimal procedures for protoplast preparation and transformation or by host restriction system (Bailey et al., 1986).

Construction of pJY713 and pJY714

To insert the tyrosinase gene (*mel*) into pJY712, pJY712 DNA was partially digested with *Bcl*I endonuclease and ligated with the tyrosinase fragment 1.5 kb of pIJ702 (Katz et al., 1983). *S. lividans* protoplasts were transformed the ligated DNA. Regenerating protoplasts were then screened for thiostrepton-resistance and melanin production by overlaying soft agar containing thiostrepton and tyrosine (500 μ g/ml). 10 out of 200 thiostrepton-resistant transformants produced the melanin pigment. 2 of these colonies were examined for plasmids and then recombinant plasmid (designated as pJY713) that inserted the tyrosinase fragment were detected by *Bcl*I digestion. And the plasmid pJY714 (7.7 kb) was constructed *in vitro* by deletion of the *Bgl*II-*Bcl*I fragment (1.9 kb) from pJY713 (Fig. 2). All transformants carrying pJY713 and pJY714 produced melanin pigment on R2YE agar (Fig. 3). Both pJY713 and pJY714 were stable in *S. lividans* without rearrangement.

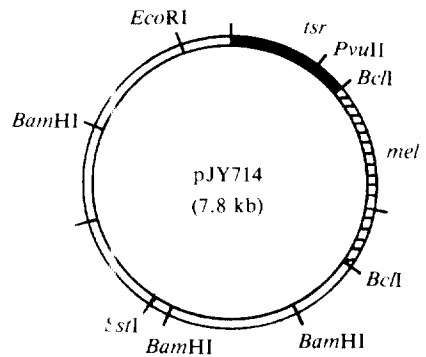
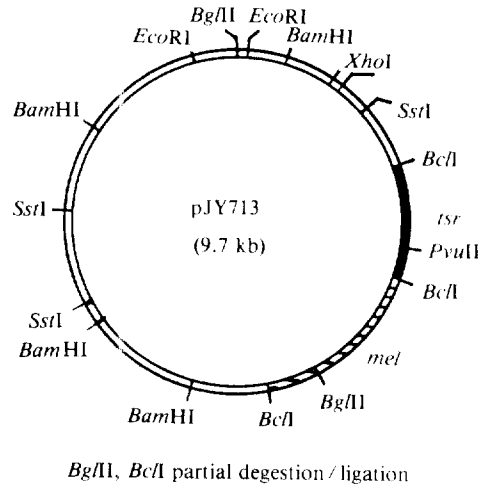


Fig. 2. Construction of recombinant plasmid pJY714.

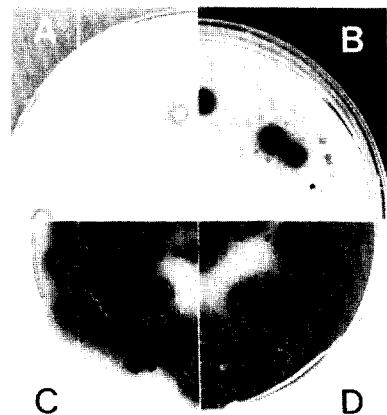


Fig. 3. Regeneration of protoplasts of *S. lividans* carrying pJY713 and pJY714 on R2YE agar after transformation.

A; Transformants carrying pJY713, B; Transformants carrying pJY714, C; Retransformants carrying pJY713, D; Retransformants carrying pJY714.

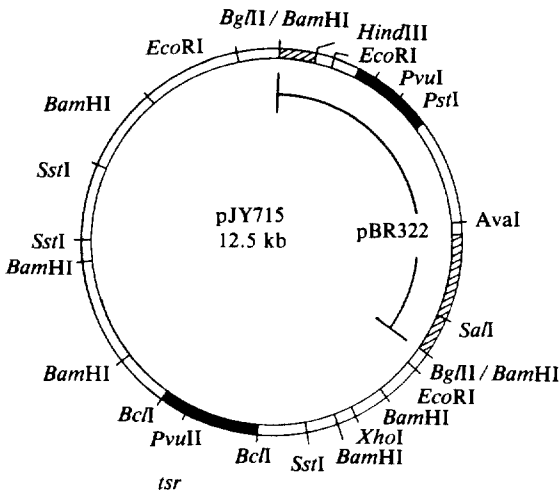


Fig. 4. Restriction map of pJY715.

Construction of bifunctional plasmids

The plasmid pBR322 was digested with *Bam* HI and ligated with *Bg*II digested pJY712. *E. coli* HB101 was transformed with each of the ligated DNA. The recombinant plasmids were found in *E. coli* HB101 by isolation of plasmid DNA of ampicillin resistant tetracycline sensitive transformants. *E. coli-Streptomyces* bifunctional plasmid, pJY715 had pBR322 in the *Bg*II site of pJY712 (Fig. 4). Transformation frequency of pJY715 was 6.2×10^3 TFU in *S. lividans*, 5.4×10^3 TFU in *E. coli*. The observation that pJY715 could transform both *Streptomyces* and *E. coli* indicated that this vector could be used to clone genes from both organisms. Also this experiment suggested that the *Bg*II site of pJY712 was not in essential region, and that *Bg*II useful for cloning widely used *Mbo*I (*Sau*3A1) was potential cloning site in pJY712.

Plasmid stability

The stability of derivatives of pJY711 was studied by counting the thiostrepton resistant colonies after 5 round of growth (spore germination, mycelial growth and sporulation) in the absence of any antibiotic pressure. The thiostrepton resistances conferred on *S. lividans* strain by the various derivatives of pJY711 were found to be inherited stable without the need for selection.

DISCUSSION

Since the first physical characterization of a *Streptomyces* plasmid (Schrempt *et al.*, 1979), several plasmids have been described in some species (Hayakawa *et al.*, 1979; Okanishi *et al.*, 1980; Nakano *et al.*, 1980). Among them, two plasmids, SCP2° and

SLP1.2, have been used as cloning vectors in *S. coelicolor*A3(2) and *S. lividans*66. But they have a low copy number (1-5 per chromosome) and a rather narrow host range. These features did not detract from the utility of SCP2° or SLP1.2 derivatives as vectors for many cloning purpose. However, in gene amplification, or the transfer of DNA between a variety of different *Streptomyces* host, plasmid vectors of higher copy number and broader host range would be useful.

We have described a naturally occurring, small and high copy-number plasmid, pJY711, from an organism classified as *Streptomyces* sp.. Even the copy number of pJY711 was lower than that of pIJ702 (Katz *et al.*, 1983), copy number 35 of pJY711 was resulted in still lower than pIJ702 (or other pIJ101 derivatives) (Schrempt *et al.*, 1975; Bibb *et al.*, 1980; Thomson *et al.*, 1980; Richardson *et al.*, 1982). Comparison of restriction enzyme pattern of the *tsr*-containing derivative demonstrated that the plasmid pJY711 was new. The small size of pJY711 and its derivatives is particularly useful since they are readily manipulated and since other pieces of DNA can be added without reaching a size too large to be stable maintained without rearrangement, in *Streptomyces*. No example of incompatibility of pJY711 with other known plasmids (pIJ702 having the replicon of pIJ101, pIJ41 having the replicon of SLP1.2) have been described. This co-existence of pJY711 with other *Streptomyces* plasmids could be useful in examining the effect of combinations of independently cloned genes within the same cell, without the need for further *in vitro* manipulation. Having a broad host range, the derivatives of pJY711 should facilitate studies on regulation and biosynthesis in some *Streptomyces* species that could not previously be extensively studied. Furthermore, these derivatives might be useful in industrial applications relating to the development and production of antibiotics. From the experiment of construction of *E. coli-Streptomyces* bifunctional plasmid, the *Bg*II site of pJY712 was proven as non-essential site for the cloning of *Mbo*I generated fragments. The recombinant plasmid pJY714 containing the *mel* fragment can be used in similar way to pIJ702 by insertional inactivation of the *mel* expression.

Because of small size, high copy number and broad host range, pJY711 and their derivatives are attractive as potential vectors for cloning in *Streptomyces*. We think that more useful vectors could be developed if experiments for allocation of nonessential regions of plasmids and size reduction are performed.

적 요

Thiostrepton 내성 유전자 (*tsr*)를 포함하는 multi-copy 재조합 플라스미드 pJY 712의 제한효소 절단지도를 작성하였다. pJY 712는 *Streptomyces*에서 넓은 host range를 나타내었으며 cloning 목적에 사용할 수 있는 단일 *Bgl* II 제한효소 인식부위를 갖고 있었다. 플라스미드 pJY 712는 lethal zygosis (Ltz⁺) 현상을 보였다. pJY 712의 형질전환빈도는 *S. lividans*에서 5.0×10^4 TFU였다. pJY 712의 *Bcl* I 제한효소 인식부위에 tyrosinase 유전자 (*mel*)를 삽입하여 플라스미드 pJY 713을 제조하였다. *mel* 유전자를 포함한 재조합 플라스미드 pJY 714는 pJY 713의 일부 (1.9 kb *Bgl* II-*Bcl* I 난편)을 제거하여 제조하였다.

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