ESTABLISHMENT OF THE HOST-VECTOR SYSTEM FOR *Micromonospora griseorubida*

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Micromonospora strains produce some important antibiotics, such as gentamicins[1], sisomicins[2], sagamicins[3], fortimicin A[4] and mycinamicins[5]. We discovered mycinamicins, 16-membered macrolide antibiotics with a strong antimicrobial activity against Gram-positive bacteria, and reported biosynthetic pathway of mycinamicin II[6]. It has been our interest to clarify the mechanisms of regulation and expression of the genes for mycinamicin biosynthesis in *Micromonospora griseorubida* in order to improve the producing strains. For this purpose, it was necessary to establish a system to express the cloned genes in *M. griseorubida*. Host-vector systems that allow to clone the antibiotic biosynthetic genes have been developed extensively in streptomycetes, but quite few systems are available in *Micromonospora*. It has been reported that *M. rosaria*, *M. purpurea* and *M. melanosporea* were transformed with the *Streptomyces* plasmids[7~9], and that some *Micromonospora* genes were cloned using the *Streptomyces* systems[10~12]. Only one host-vector system for *Micromonospora* using *Micromonospora* plasmids was reported[13].

Here, we describe construction of novel plasmids and improvement of transformation efficiency to establish the host-vector system of *M. griseorubida*.

In our preliminary experiments, no streptomycete plasmids transformed *M. griseorubida* A11725 stably. Therefore, we screened for plasmids in a number of strains of *Micromonospora*. Consequently, a cryptic plasmid p11725a (Fig. 1) was discovered in *M. griseorubida* A11725 and used to construct vectors for *M. griseorubida*.

For plasmid isolation and protoplast formation, *M. griseorubida* A11725 grown on M agar consisting of soluble starch 20.0 g, Polypepton 5.0 g, $CaCO_3$ 1.0 g, $FeSO_4 \cdot 7H_2O$ 40 mg, soy bean meal ("Esusanmito"; Ajinomoto Co.) 7.5 g and Bacto-agar (Difco) 22 g, per liter of deionized water was subsequently incubated in 172F medium[14]. Ten ml of 172F medium was inoculated with a loopful from a slant culture of *M. griseorubida* A11725 and shaken at 28°C for 44 hours at 200 rpm. A 2.5 ml of aliquot of this seed culture was transferred to 50 ml of 172F medium containing 0.2% of glycine, and incubated at 28°C for 48 hours on a rotary shaker at 200 rpm. Mycelia were collected by centrifugation and washed once with 10 ml of 0.3 M sucrose. The resulting pellet was resuspended in 10 ml of L medium[15] containing 2 mg/ml lysozyme (Seikagaku Kogyo Co.) and 1 mg/ml achromopeptidase (Wako Pure Chemical Industries Co.), and incubated at 37°C for 2~3 hours. Protoplasts were filtered through cotton wool, centrifuged and resuspended in 1 ml of P medium[15]. To obtain a good yield of protoplasts, growth phase turned out to be effective as pointed out in *Streptomyces*[16]. A dramatic increase in yield was observed using mycelia for 44 hours incubation (early stationary phase). While the number of protoplasts obtained from 36 hours incubated culture was 10^8/ml, that from 44 hours culture provided 10^{10}/ml.

For regeneration of *M. griseorubida* protoplasts, R2YE[17] generally used for regeneration of *Streptomyces* protoplasts, gave the regeneration frequency as low as <0.1%. Therefore, we designed MR0.3S by modifying the M agar, which was used for the cultivation of *M. griseorubida*. MR0.3S medium contained sucrose 103.0 g, soluble starch 20.0 g, soy bean meal 7.5 g, Polypepton 5.0 g, $CaCO_3$ 1.0 g, $MgSO_4 \cdot 7H_2O$ 5.0 g, trace elements solution[15] 10.0 ml, $FeSO_4 \cdot 7H_2O$ 40.0 mg and Bacto-agar 22.0 g, per liter of deionized water (adjusted to pH 7.2 with 1 N NaOH). Soft agar containing sucrose 103.0 g, $MgCl_2 \cdot 6H_2O$ 10.1 g, $CaCl_2 \cdot 2H_2O$ 3.0 g, Bacto-agar 6.5 g and 0.25 M TES pH 7.2 100.0 ml, per liter of deionized water was overlaid for spreading the protoplasts. 6.2% of *M. griseorubida* A11725 protoplasts consequently regenerated on the medium. The new regeneration medium MR0.3S worked well not only for regeneration of

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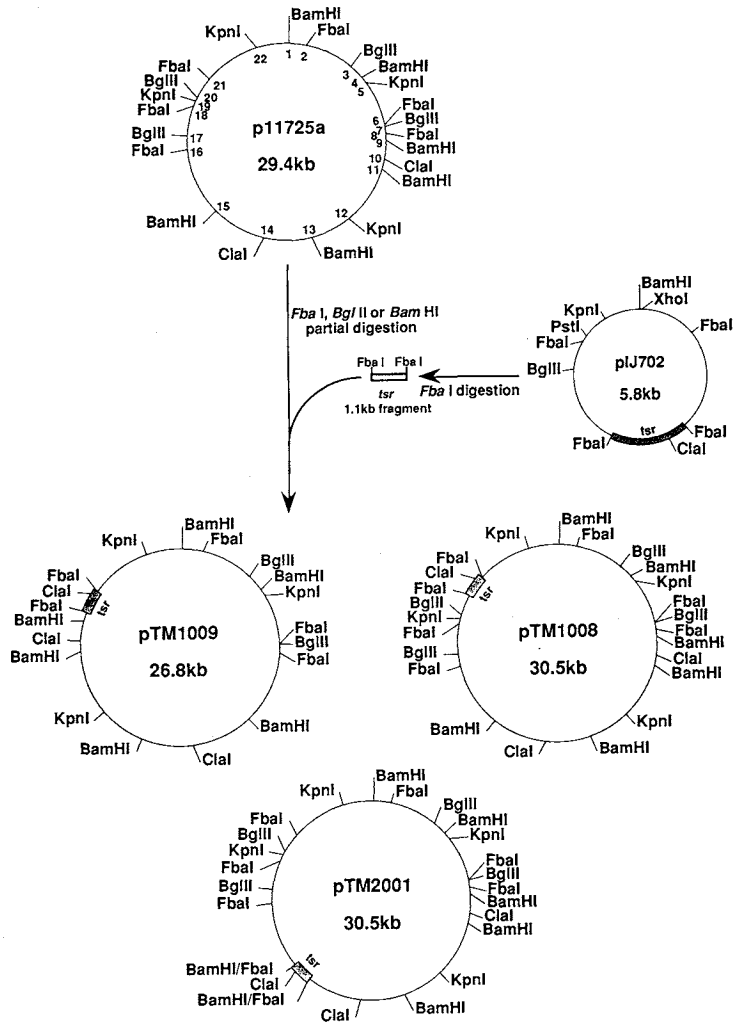
Fig. 1. Construction of plasmid vectors for *Micromonospora griseorubida*.



M. griseorubida protoplasts but also for that of *Streptomyces* protoplasts[18].

Vectors were constructed as follows. Strain A11725 was highly resistant not only to mycinamicin II (1500 μg/ml) but also to hygromycin B (>200 μg/ml), while was sensitive to neomycin (2 μg/ml), thiostrepton (2 μg/ml) and viomycin (50 μg/ml). We chose thiostrepton-resistance gene (*tsr*) as a selective marker, because thiostrepton-resistant transformants of *M. griseorubida* were obtained transiently with *Streptomyces* plasmids such as pIJ702[19] and pIJ922[20] at low frequency. The scheme of vector construction was shown in Fig. 1. p11725a was prepared as described by KIESER[21] and purified by CsCl-ethidium bromide centrifugation[22]. Two μg of the purified p11725a was partially digested with *Fba*I, *Bgl*II or *Bam*H I, and ligated with the 1.1 kb *Fba*I fragment containing the *tsr* gene from pIJ702. All of the ligation mixture (50 μl) was added with 200 μl of protoplast suspension ($1 \times 10^9 \sim 5 \times 10^9$) followed by 500 μl of T medium[23] containing 25% polyethylene glycol (PEG) 1000 (Nakarai Tesque, Inc.) and mixed by gentle pipetting. The solution was allowed to stand for 60 seconds at room temperature and added with 500 μl of P medium to terminate transformation. After mixing by pipetting, an aliquot of this suspension was spread with 2.5 ml of soft agar kept at 42°C onto MR0.3S agar plate (25 ml). After subsequent incubation at 28°C for 4 days, the regeneration plates were overlaid with 2.5 ml of soft agar containing 200 μg/ml thiostrepton (Sigma) and further incubated at 28°C for 2~3

Table 1. Stability of plasmids in *Micromonospora griseorubida*.

Plasmids	Number of colonies/plate		Thio$^+$/thio$^-$ (%)
	thio$^+$	thio$^-$	
pTM1009	432	475	90.9
pTM1008	427	477	89.5
pTM2001	5	404	1.2

M. griseorubida transformants containing pTM1009, pTM1008 or pTM2001 were inoculated into 10 ml of 172F medium without thiostrepton and incubated at 28°C. After 3 days, whole broth was homogenized and 0.1 ml of the culture was transferred into 10 ml of fresh 172F medium. After this procedure was repeated 5 times, cultures were homogenized, diluted and spread onto M agar with or without thiostrepton. After incubation at 28°C for 7 days, arising colonies were counted.

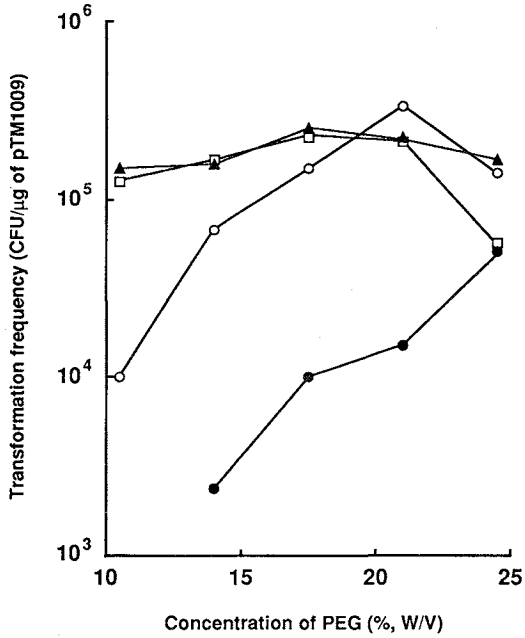
weeks. The thiostrepton-resistant colonies were picked on the M agar containing 20 μg/ml thiostrepton to confirm resistance.

pTM1009, pTM1008 and pTM2001 (Fig. 1) were recombinant plasmids prepared from the thiostrepton-resistant colonies. In pTM1008 and pTM2001, the *tsr* gene was inserted at *Fba*I site 21 and *Bam*H I site 15, respectively. In pTM1009, two *Fba*I fragments between sites 16~21 of p11725a were lost and the *tsr* gene was inserted. Furthermore, the largest *Fba*I fragment between sites 8~16 was rejoined in the opposite direction. As shown in Table 1, pTM1008 and pTM1009 were stably maintained relatively, even though in the absence of thiostrepton, but pTM2001 was not stable in *M. griseorubida*. The structural difference between pTM1008 and pTM2001 was the insertion site of the *tsr* gene, suggesting that the *Bam*H I site (site 15 in p11725a) might be located in the region relating to the plasmid stability. Furthermore, deletion analysis indicated that replication region of p11725a was located between *Fba*I site 8 and *Kpn*I site 12, and *Fba*I fragment (between site 8 and site 16) was essential for construction of the stable vectors from p11725a (data not shown).

To improve the host-vector system, we examined the conditions of regeneration and transformation using pTM1009. It turned out that when the concentration of sucrose as an osmotic stabilizer in regeneration medium and soft agar was reduced from 0.3 M to 0.1 M, the regeneration frequency and the size of regenerated colonies increased remarkably (from 6.2% to 16.3%, 2 times in diameter). Substituting $CaCO_3$ for TES buffer (25 mM, pH 7.2) yielded additional stimulative effect on regeneration

Fig. 2. Effects of PEG on transformation frequency of *Micromonospora griseorubida*.

● PEG 1000, ○ PEG 2000, ▲ PEG 4000, □ PEG 6000.



and the medium thus established was designated as MR0.1S. In addition, about 15% dehydration of the surface of the regeneration plate[24] gave the best regeneration frequency (49.0%).

Subsequently, we tested the effects of molecular weight and concentration of PEG on transformation frequency. PEG 2000, 4000 and 6000 gave better results than PEG 1000 which was used for the initial study (Fig. 2). This result showed that PEG 1000 was rather toxic on transformation of *M. griseorubida* among the PEG tested. The highest transformation frequency (3.4×10^5 CFU/μg of pTM1009) was obtained at 21% (final concentration) of PEG 2000 and was about 30 times increase, compared with that of the initial study (at 17.5% of PEG 1000).

Efficient transformation system in *M. griseorubida* A11725 was established in this study. It was obtained by constructing novel plasmids stably maintained in *Micromonospora*, and design of the new regeneration media. Using the smallest and stably maintained plasmid pTM1009, we would construct more useful vectors to transduce large DNA fragments to *M. griseorubida* for cloning of the mycinamicin biosynthetic genes and to improve the mycinamicin producing strains.

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