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Conjugal Transfer of Plasmid DNA from *Escherichia coli* to *Streptomyces lavendulae* FRI-5

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Abstract *Streptomyces lavendulae* FRI-5 produces the γ-butyrolactone autoregulator IM-2, which is required for nucleoside antibiotic production. We have developed a system for introducing DNA into *S. lavendulae* FRI-5 *via* conjugal transfer from *Escherichia coli*. Conditions were established for conjugation of the *oriT*- and *attP*-containing plasmid pSET152 from *E. coli* ET12567 (pUZ8002) to FRI-5. Conjugation resulted in integration of the plasmid at the chromosomal ϕC31 *attB* site. The frequency of intergeneric conjugation varied with the medium used. The highest frequency (1.6×10^{-5} per recipient) was obtained on ISP medium 2 containing 10 mM $MgCl_2$. Southern blot and phenotypic analyses of exconjugants revealed that *S. lavendulae* FRI-5 contains a unique ϕC31 *attB* site, and that integration of heterologous DNA into the *attB* site did not interfere with morphological differentiation or IM-2-dependent signal transduction, including the production of a blue pigment. This system will now enable detailed genetic analysis of the regulation of antibiotic production in *S. lavendulae* FRI-5.

Key words: *Streptomyces*, conjugation, integrating plasmid, secondary metabolite

Streptomyces species are versatile producers of many secondary metabolites, including over two-thirds of all antibiotics used in human medicine and in agriculture [1, 2]. In recent years, there has been a rapid accumulation in our understanding of the mechanisms that regulate antibiotic production and morphological differentiation in streptomycetes [9]. The most significant step in acquiring this knowledge has been the establishment of mechanisms for introducing DNA into these organisms. This has permitted functional gene cloning, gene disruption, and mutant complementation. Although transformation of *Streptomyces* protoplasts has been achieved with a number of quite different species [8], the procedure established for one strain is not always readily applicable to another streptomycete. Recently, there has been a considerable increase in the use of intergeneric conjugation from *Escherichia coli* to *Streptomyces*, which has the added benefit that it can assist in circumventing often potent restriction barriers [12].

Streptomyces lavendulae FRI-5 produces the anti-tuberculosis antibiotic D-cycloserine during early growth [15, 25], but switches to produce the nucleoside antibiotics showdomycin and minimycin [6] and a blue pigment [25] later in growth. This switching mechanism requires an extracellular regulatory factor, IM-2 [21], which belongs to the family of γ-butyrolactone autoregulators found in many *Streptomyces* species [10]. The IM-2-specific receptor protein (FarA) was purified and characterized [19], and the gene (*farA*) encoding it cloned and analyzed [11, 24]. The results indicate that FarA is likely to act as a transcriptional repressor in the signal transduction pathway that triggers secondary metabolism. However, understanding the *in vivo* function of FarA in *S. lavendulae* has been hindered by the lack of a means to introduce DNA into the strain. In this study, we established a simple and reliable procedure for the conjugal transfer of DNA from *E. coli* to *S. lavendulae* using the *oriT*- and *attP*-containing plasmid, pSET152. Phenotypic analysis of exconjugants revealed that integration of heterologous DNA into the ϕC31 *attB* site has no deleterious effect on IM-2-dependent gene regulation, thus providing a viable system for analyzing the regulation of secondary metabolism in this organism.

Bacterial Strains, Growth Conditions, and Plasmids

S. lavendulae FRI-5 (MAFF10-06015; National Food Research Institute, Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Japan) was used as a recipient throughout this study. The methylation-deficient *Escherichia coli* strain ET12567 (*dam-13*::Tn9, *dcm-6*, *hsdM*, *hsdS*) [13] containing pUZ8002 was used as the donor in intergeneric conjugations. pUZ8002 [18] is a RK2 derivative with a

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defective *oriT* (*aph*): it is not self-transmissible but supplies mobilization functions for *oriT*-containing plasmids *in trans*. Procedures for standard DNA manipulations in *E. coli* and *Streptomyces* were as described in [20] and [8], respectively.

Effect of Heat Treatment on the Viability of *S. lavendulae* FRI-5 Spores

To effect intergeneric plasmid transfer from *E. coli* to streptomycetes, recipient spores are often subjected to heat treatment (for instance, 50°C for 10 min for *Streptomyces coelicolor* A3(2) [8]) before being mixed with *E. coli* donor cells; this is because spore germination was thought to be necessary for efficient conjugation [4]. Thus, the viability of *S. lavendulae* FRI-5 spores after different heat treatments was first assessed. While spores incubated at 50°C quickly lost viability (Fig. 1A), incubation at 40°C or lower for 10 min had little effect (Fig. 1B). Phase contrast microscopy confirmed that incubation at 40°C for 10 min induced spore germination. Consequently, a 10 min incubation at 40°C was used in all subsequent conjugation experiments.

Intergeneric Conjugation

Before attempting intergeneric conjugation, the presence of a suitable ϕC31 *attB* site(s) in the *S. lavendulae* FRI-5 genome was assessed by infecting the strain with the ϕC31 derivatives, KC212 and KC213. These phages contain the ϕC31 *attP* and *int* functions, and the *apr* gene, which confers resistance to apramycin [Bruton, C. and K. F. Chater, Personal communication]. Both phages yielded apramycin resistant lysogens, indicating that *S. lavendulae* possessed a ϕC31 *attB* site(s) that should also be available for site-specific integration by suitable conjugative plasmids.

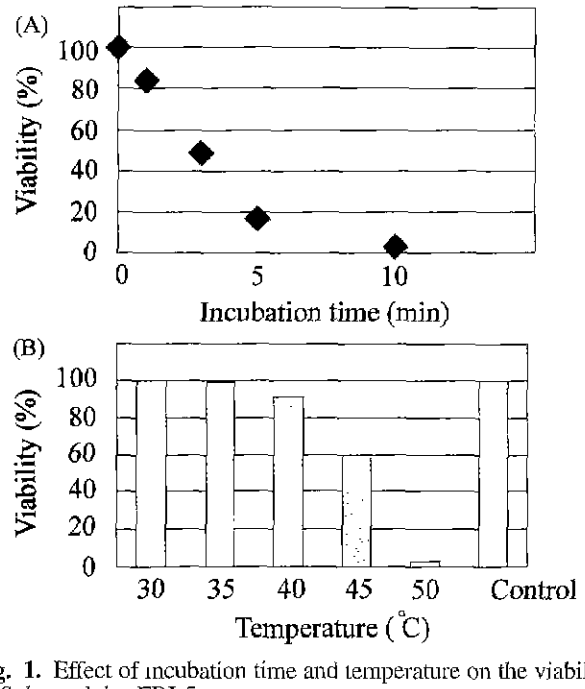

Fig. 1. Effect of incubation time and temperature on the viability of *S. lavendulae* FRI-5 spores.

(A) A spore suspension of *S. lavendulae* FRI-5 was diluted to 10^5 spores/ml, and incubated at 50°C for the time indicated. (B) Spores (10^7/ml) in 2 x YT medium were incubated for 10 min at the temperature indicated. *Viability was determined by counting colonies on* ISP2 *medium* after incubation at 28°C for 7 days. The data are the average of two independent experiments, and expressed as a percent of the colony count obtained in the absence of heat treatment.

For conjugal transfer from *E. coli* [16], we selected *E. coli* ET12567 harboring pUZ8002 as donor, and pSET152 [3] and pPM925 [22] as the conjugative plasmids. Since neither plasmid possesses an origin of replication that is functional in streptomycetes, but both plasmids contain *oriT* and the ϕC31 *attP* and *int* genes which direct site-specific integration of the plasmids into the streptomycete chromosomal *attB* site, they can only exist in *S. lavendulae* FRI-5 as chromosomally integrated forms. Spores of *S. lavendulae* FRI-5, with or without heat-treatment, were mated with *E. coli* ET12567 (pUZ8002) harboring either pSET152 or pPM925. While pSET152 gave large numbers of exconjugants (Table 1), pPM925 gave none under any conditions. This may reflect the larger size of pPM925 (11.4 kb compared to the 5.5 kb of pSET152). An effect of plasmid size on the efficiency of intergeneric conjugation was also reported by Flett *et al.* [4] for *S. coelicolor* A3(2). The efficiency of pSET152 transfer varied markedly with the medium used: MS agar (mannitol plus soya flour) [7] containing 10 mM $MgCl_2$ consistently gave frequencies of about 10^{-6} exconjugants per recipient regardless of the recipient/donor ratio. ISP2 containing 10 mM $MgCl_2$ gave 1.6×10^{-5} to 3.9×10^{-8} exconjugants per recipient, with higher conjugation frequencies at lower recipient/donor ratios. R5 agar [8] gave no transconjugants. Unlike *S. coelicolor* A3(2) [4] or *Streptomyces toyocaensis* [14], germination of recipient spores prior to mixing with the *E. coli* donor did not increase the frequency of conjugation. To confirm chromosomal integration of pSET152, exconjugants were analyzed by Southern blot hybridization (data not shown). All of the exconjugants showed identical hybridization patterns, suggesting that *S. lavendulae* FRI-5 contains a

Table 1. Conjugal transfer of pSET152 from *E. coli* ET12567 (pUZ8002) to *S. lavendulae* FRI-5.

Heat treatment[a]	Number of recipient spores	Exconjugants per recipient[b]		
		MS+$MgCl_2$	ISP2+$MgCl_2$	R5
-	10^{10}	9.7×10^{-6}	7.0×10^{-8}	-[c]
	10^9	6.3×10^{-6}	5.4×10^{-7}	-
	10^8	3.8×10^{-6}	1.6×10^{-5}	-
+	10^{10}	8.8×10^{-6}	3.9×10^{-8}	-
	10^9	4.5×10^{-6}	4.6×10^{-7}	-
	10^8	2.4×10^{-6}	1.4×10^{-5}	-

[a]Conditions of heat treatment were at 40°C for 10 min.
[b]Values represent average frequencies from two independent experiments.
[c]- indicates no exconjugants.

unique ϕC31 *attB* site and indicating that pSET152 integrates into this site.

Plasmid Integration has No Effect on Phenotype

The presence of heterologous DNA in streptomycetes, either chromosomally integrated or plasmid-borne, has sometimes resulted in a reduction in antibiotic productivity and/or a reduction in growth [23]. However, the *S. lavendulae* FRI-5 exconjugants containing pSET152 grew at the same rate as the wild-type strain in liquid culture and produced similar levels of antibiotic, which were determined by plate assays with *Bacillus subtilis* PCI219 [17]. They were also indistinguishable from the wild-type strain in their morphological characteristics on a range of different solid media, such as ISP2, oatmeal agar [6], MS agar, MM agar plus mannitol, R2 agar [8], and SMMS agar [5]. Thus, integration of DNA at the ϕC31 *attB* site appears to be neutral with regard to both morphological differentiation and antibiotic production. To further assess whether plasmid integration might have influenced the IM-2 signal transduction cascade, the ability of IM-2 to induce the production of blue pigment and the binding activity of the IM-2 receptor protein FarA was determined in exconjugants and their parental strain [6, 19]. All of the exconjugants showed induction of blue pigment production 2 h after the addition of IM-2 to a 5-h liquid culture, as in the parental strain. Moreover, the IM-2 binding activity in crude-cell extracts of both strains was essentially identical (IM-2 binding activity per mg of protein: parental strain, 2.21 pmol; exconjugant, 2.08 pmol).

We have established a system for the intergeneric transfer of DNA from *E. coli* ET12567 (pUZ8002) to *S. lavendulae* FRI-5, and confirmed that chromosomal integration does not influence the traits that we wish to study in this strain. More importantly, this procedure will allow targeted gene disruption and mutant complementation in *S. lavendulae* FRI-5, and allow us to investigate the complex regulatory cascade of IM-2-dependent regulation of secondary metabolism in this strain.

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