Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp.

(Recombinant DNA; phage φC31; gene disruption; shuttle vectors; cosmids; P1 vector; mobilization; gene transplacement; SCP2*)


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Received by C.R. Hutchinson: 12 November 1991; Revised/Accepted: 6 February 1992; Received at publishers: 11 March 1992

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**SUMMARY**

We have constructed cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. All vectors contain the 760-bp oriT fragment from the IncP plasmid, RK2. Transfer functions need to be supplied in trans by the *E. coli* donor strain. We have incorporated into these vectors selectable antibiotic-resistance markers (AmR, ThR, SpR) that function in *Streptomyces* spp. and other features that should allow for: (i) integration via homologous recombination between cloned DNA and the *Streptomyces* spp. chromosome, (ii) autonomous replication, or (iii) site-specific integration at the bacteriophage φC31 attachment site. Shuttle cosmids for constructing genomic libraries and bacteriophage P1 cloning vector capable of accepting approx. 100-kb fragments are also described. A simple mating procedure has been developed for the conjugal transfer of these vectors from *E. coli* to *Streptomyces* spp. that involves plating of the donor strain and either germinated spores or mycelial fragments of the recipient strain. We have shown that several of these vectors can be introduced into *Streptomyces fradiae*, a strain that is notoriously difficult to transform by PEG-mediated protoplast transformation.

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**INTRODUCTION**

*Streptomyces* are known for their ability to synthesize commercially useful secondary metabolites with a variety of biological activities (Deshpande et al., 1988; Hopwood, 1989). The macrolide antibiotics tylosin (produced by *S. fradiae*) and spiramycin (produced by *Streptomyces ambofaciens*) are two examples. We have been interested in the genetics of tylosin and spiramycin biosynthesis, and for that purpose have cloned the tylosin and spiramycin biosynthetic gene clusters from *S. fradiae* (Baltz and Seno, 1988; Fishman et al., 1987) and *S. ambofaciens* (Richard-
son et al., 1990), respectively. This work was carried out with available vectors that were introduced into Streptomyces spp. by PEG-mediated protoplast transformation. This procedure was not very satisfactory for S. fradiae even with non-restricting mutants (Matsushima et al., 1985; Matsushima et al., 1987) because of low transformation frequencies with plasmid DNA isolated from E. coli, and because of the time and labor required to prepare protoplasts (Rao et al., 1987). Inter-generic transfer of plasmids from E. coli to Streptomyces spp. was demonstrated initially by Mazodier et al. (1989), who incorporated oriT from RK2 into a replicating plasmid pPM803, and subsequently by Smokvina et al. (1990), who constructed PSAM2-based integrative vectors that incorporated the oriT region and could be conjugally transferred from E. coli to Streptomyces spp.

To determine if conjugal transfer of plasmids would facilitate genetic analysis of S. fradiae, we constructed different kinds of conjugal vectors, including those which can integrate by homologous recombination into the genome, those which can replicate autonomously, and those which can integrate site-specifically using the bacteriophage φC31 integration functions. Mating conditions are described for the introduction of these vectors into S. fradiae and other Streptomyces spp.

RESULTS AND DISCUSSION

(a) Plasmid constructions

Table I lists plasmids described in this report. They represent three different types: non-replicating plasmids (Fig. 1), replicating plasmids (Fig. 2) and integrating plasmids (Fig. 3). They all contain the 760-bp oriT fragment from RK2 (Guiney and Yakobson, 1983) and E. coli replication functions from pUC (Yanisch-Perron et al., 1985), P15A (Chang and Cohen, 1978), or P1 (Sternberg, 1990). Thus, these plasmids serve as cloning vectors for constructions to be made in E. coli, which subsequently can be transferred by conjugation to the desired Streptomyces spp. recipients.

Fig. 1 shows four plasmids (pOJ260, pSET151, pKC1138 and pKC1250) that do not replicate in Streptomyces spp., and thus they should be useful for gene disruption and gene transplacement experiments (Kuhstoss et al., 1989; Muth et al., 1989; Richardson et al., 1990). For cloning purposes in E. coli, these plasmids contain MCS within lacZa which allows for easy detection of recombinant clones (Sambrook et al., 1989). Selectable markers conferring AmR or SpR were incorporated into pOJ260 (AmR), pKC1138 (AmR) and pKC1250 (SpR) because they function in E. coli and Streptomyces spp. (Kuhstoss et al., 1991; Howell and Rao, 1992). The ApR marker for selection in E. coli and the ThR marker for selection in Streptomyces spp. were incorporated into pSET151. Plasmid pKC1250 was constructed to facilitate gene disruption experiments in strains already containing integrated pUC-based plasmids. The use of the P15A-derived replicon as in pKC1250 will prevent recombination between homologous vector sequences.

To simplify the screening for segregants that have undergone gene transplacement, we inserted the xylE reporter gene into pKC1138 and pSET151, which is driven by a synthetic promoter in pKC1138 and by the ermE promoter in pSET151. Although the xylE reporter gene from Pseudomonas has been used in several Streptomyces strains (Zukowski et al., 1983; Ingram et al., 1989; Guthrie and Chater, 1990; Bruton et al., 1991; Kuhstoss and Rao, 1991), its expression in S. fradiae, when encoded by a single gene copy, can be somewhat variable. Nevertheless, we have used this reporter gene successfully for phenotypic discrimination between xylE+ and xylE- to screen for plasmid loss.

The plasmids shown in Fig. 2 contain replication functions for Streptomyces spp. and thus they can exist as autonomous, multicopy plasmids. These plasmids can be used for complementing mutations, increasing the copy number of cloned genes and allele replacements. Plasmid pKC1218 was derived from pOJ260 (Fig. 1) by inserting the SCP2* replicon (Larson and Hershberger, 1984; 1986). Plasmid pKC1139 contains a temperature-sensitive replicon from

### Table I

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Size (kb)</th>
<th>Relevant characteristics</th>
<th>Derivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-replicatinga</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pOJ260</td>
<td>3.5</td>
<td>AmR reppUC</td>
<td>See Fig. 1</td>
</tr>
<tr>
<td>pSET151</td>
<td>6.2</td>
<td>ThR ApR xylE+ reppUC</td>
<td>See Fig. 1</td>
</tr>
<tr>
<td>pKC1132</td>
<td>3.5</td>
<td>AmR reppUC</td>
<td>pOJ260; deletion of KpnI to SpeI sites</td>
</tr>
<tr>
<td>pKC1138</td>
<td>6.6</td>
<td>AmR xylE+ reppUC</td>
<td>See Fig. 1</td>
</tr>
<tr>
<td>pKC1250</td>
<td>3.8</td>
<td>SpR repSCP2</td>
<td>See Fig. 1</td>
</tr>
<tr>
<td>Integratingb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pSET152</td>
<td>5.5</td>
<td>AmR lacZa MCS repUC</td>
<td>See Fig. 3</td>
</tr>
<tr>
<td>pOJ436</td>
<td>1.1</td>
<td>AmR (cos3') repSCP2</td>
<td>See Fig. 3</td>
</tr>
<tr>
<td>pOJ444</td>
<td>35.3</td>
<td>AmR KnR repF' repP11</td>
<td>See Fig. 3</td>
</tr>
<tr>
<td>pKC1116</td>
<td>10.1</td>
<td>AmR (cos3') repA'</td>
<td>pOJ436 AarII deletion</td>
</tr>
<tr>
<td>pKC1163</td>
<td>5.5</td>
<td>AmR lacZa MCS repSCP2</td>
<td>See Fig. 3 legend</td>
</tr>
</tbody>
</table>

a Additional features present in these plasmids are a MCS in lacZa and oriT.
b Additional features present in these plasmids are oriT and reppUC.
't Additional features present in these plasmids are oriT and intSCP2.
S. ghanaensis (Muth et al., 1989) which functions only at temperatures below 34°C. Thus, this plasmid constitutes a useful delivery system for transposons (Solenberg and Baltz, 1991) and for experiments requiring homologous recombination between plasmid-borne sequence and the chromosome. The cosmid vector pOJ446 contains the SCP2* replicon and is capable of accepting up to approx. 35-kb DNA segments. The BamHI cloning site in pOJ446 is flanked by two restriction sites, XbaI and SpeI, that are rare in Streptomyces spp., thus allowing for easy removal of the intact cloned DNA. The T3 and T7 bacteriophage promoters were added to facilitate transcriptional analyses (Loewy et al., 1989).

Plasmids and cosmids that can integrate site-specifically at the φC31 attachment site are shown in Fig. 3. They give rise to stable exconjugants that can be propagated without detectable loss of plasmid markers, even in the absence of drug selection (E. T. S., unpublished). The plasmid vectors contain the AmR marker for selection and lacZ+ with MCS for cloning DNA. The cosmid vectors contain three cos sites (Rao et al., 1987; Richardson et al., 1987) for in vitro DNA packaging. Unlike the non-replicating vectors, these vectors can be used to insert homologous or heterologous DNA into the chromosome at the unique attBS site (Kuhstoss and Rao, 1991). The φC31-driven, site-specific recombination is apparently very efficient because plasmids containing as much as 8 kb of homologous DNA were found only at the φC31 attBS site, with no detectable integration by homologous recombination (E. T. S., unpublished).

An E. coli φC31 using vector (pOJ444) utilizing the bacteriophage P1 packaging system has also been constructed, which potentially could be used for cloning DNA fragments of approx. 100 kb (Sternberg, 1990; 1992). Although this plasmid has been shown to integrate site-specifically at
Fig. 2. Restriction maps of pKC1139, pKC1218 and pOJ446. Plasmid pKC1139 was derived from pOJ260 (Fig. 1). The 2.9-kb region between the Clal site and oriT containing the temperature sensitive replicon from pSW344E was excised with PndI+Xhol (Muth et al., 1989), and inserted between the Kpnl and Spel sites in pOJ260 after treatment with T4 DNA polymerase. Plasmid pKC1218 is a derivative of pOJ260 (Fig. 1). The 2.2-kb BamH! fragment from pHJL366 (Larson and Hershberger, 1986) containing the SCP2* replicon was inserted at the Bglll site (BamHI/Bglll indicates the two junctions) in pOJ260. Plasmid pOJ446 was derived from pKC505 (Rao et al., 1987) by a series of steps. The unique BamH! site can be used to insert DNA fragments, and subsequent cutting a Pvull or Hpal generates the two arms used in cosmid cloning (Rao et al., 1987). To generate pOJ446, oriT was isolated from pPM803 (Mazodier et al., 1989) on a Pstl fragment which was inserted at a Pstl site. Asterisks indicate known unique restriction sites in the lacZ~ MCS in plasmids pKC1139 and pKC1218.

(b) Conjugal transfer of vectors from Escherichia coli and Streptomyces spp.

Conjugal transfer of DNA from E. coli to Streptomyces spp. requires the 760-bp oriT fragment and transfer functions supplied in trans by the E. coli donor strain S17-1 (Mazodier et al., 1989; Simon et al., 1983). Most of our experiments were done using S. fradiae as the conjugal recipient, because PEG-mediated protoplast transformation of S. fradiae occurs with very low efficiency, particularly when large plasmids or cosmids isolated from E. coli are used. Although highly transformable strains of S. fradiae have been isolated following several rounds of mutagenesis, these strains were constructed in tlyc non-producers (Matsushima et al., 1987) and appear to have acquired additional mutations that limit their use. Another strain, Sa. spinosa (Mertz and Yao, 1990), was refractory to PEG-mediated transformation and the only successful means of introducing DNA was by conjugal transfer from E. coli.

Conditions used for conjugal matings between E. coli and S. fradiae are given in the legend to Fig. 4. The original protocol for E. coli-Streptomyces spp. conjugal matings reported by Mazodier et al. (1989) used freshly germinated spores of the streptomycete as the conjugal recipient. We have used this protocol successfully, but in order to accommodate actinomycetes that sporulate poorly (or not at all) and to expedite the preparation of the recipient culture, we have developed a protocol using vegetative mycelium rather than spores. Using pSET152 (Fig. 3), we have reproducibly achieved exconjugant frequencies of 10-15% of the initial viable recipient population using either germinated spores or vegetative mycelia. Similar results were obtained with pSET169 (not shown) which contains the xylE gene driven by the paf promoter (E. T. S., unpublished). Although exconjugants have been obtained from matings carried out at temperatures between 29°C and 37°C, 37°C appears to be optimal, yielding about three times as many exconjugants as obtained at 29°C. Matings done on AS 1 medium (Baltz, 1980) generated exconjugants at a frequency 1000-fold greater than on TS agar; no exconjugants were obtained on modified R2 medium (Baltz, 1978).

Plasmid size affected conjugation frequencies. In general, the frequency was much higher (100-1000 times) with small plasmids (approx. 5.5 kb) than with large (approx. 20 kb) plasmids or cosmids (45-50 kb). However, the pre-
cise numbers varied with the type of plasmid used and the size and source of the DNA insert. This is not surprising because recipients are expected to restrict incoming DNA to varying degrees, depending on the frequency and sequence context of susceptible restriction sites.

*S. ambofaciens* can be readily transformed with plasmids isolated from *E. coli* (Matsushima and Baltz, 1985), but introduction of DNA by conjugal transfer provided an alternative and easier method. Most of the conditions and variables described for *S. fradiae* apply to *S. ambofaciens*. Plasmids with the oriT region could be mobilized, not only from *E. coli* S17-1 where RP4 is chromosomally located, but also from *E. coli* strains in which RP4 is free in the cytoplasm. With *Sa. spinosa*, we found that the mating conditions described yielded exconjugants, although the frequencies were considerably lower (1–200 per mating plate) compared with *S. fradiae* or *S. ambofaciens*.

Using these conditions and the vectors described in the report, we have created gene disruptions and gene replacements within the tylosin and spiramycin gene clusters in *S. fradiae* and in *S. ambofaciens* (in preparation), increased the gene dosage of the tylF gene in *S. fradiae* (E.T.S., in preparation), and introduced large (approx. 35 kb) homologous or heterologous DNA fragments on cosmid vectors into *S. fradiae*, *S. ambofaciens* and *Sa. spinosa* (M.B., unpublished).

(c) Conclusions
(1) Non-replicating, freely replicating and integrating plasmid or cosmid vectors were constructed and shown to transfer efficiently from *E. coli* to *Streptomyces* by conjugation.

(2) The highest numbers of *S. fradiae* exconjugants were obtained in matings carried out at 37°C on AS1 agar plates using either freshly germinated spores or early exponential phase mycelial fragments as the conjugal recipient.
Fig. 4. Conjugal transfer of plasmids from E. coli to Streptomyces. One ml of a frozen mycelial culture of S. fradiae was diluted into 9 ml of TS broth (Baltz, 1978) and grown for 18 h aerobically at 29°C. The culture was homogenized (Baltz, 1978) and 2 ml was transferred into 18 ml of fresh TS broth and grown for 16 h at 29°C to obtain a late log phase culture. This culture was homogenized and fragmented with ultrasound (Baltz, 1978), and 1 ml was transferred to 9 ml TS broth. The culture was incubated aerobically at 37°C for 3 h. The mycelium was recovered by centrifugation, washed once in TS broth and resuspended in 2 ml TS broth (recipient culture). The E. coli donor, S17-1[pSET169], was grown at 37°C overnight in TY broth plus 100 µg Am/ml, subcultured 1:100 and grown for 3 h at 37°C. The cells were pelleted, washed once in TS broth and resuspended in 2 ml TS broth (donor culture). Equal vols of the donor culture and ten-fold serial dilutions of the recipient culture were mixed, and 100 µl were plated to ASI (Baltz, 1980), supplemented with 10 mM MgCl₂. Plates were incubated at 37°C for 16 h, and then covered with 3-4 ml of soft R2 agar (Baltz, 1980) containing 1.5 mg nalidixic acid and 1.5 mg of Am. Incubation at 37°C was continued for about a week to allow outgrowth of the exconjugants. a, 50 µl of the recipient culture alone selected for Am resistance (negative control); b, 10 µl of recipient culture, no Am; c, 10 µl of a 10⁻⁵ dilution of the recipient culture, no Am; d, Am⁺ exconjugants obtained when undiluted recipient was used; e, Am⁺ exconjugants obtained when 10⁻⁵ diluted recipient was used. No colonies grew when 100 µl of donor culture was plated with Am and nalidixic acid. Incubation at 34°C instead of 37°C gave similar results.

ACKNOWLEDGEMENTS

We are particularly thankful to P. Mazodier, R. Petter, C. Thompson and J. Davies for sharing pPM801 and pPM803 plasmids and E. coli S17-1 strain with us, without which this work could not have been done. We thank M. Howell, M. Richardson, S. Kuhstoss, M. Basinski and F. Laquier for sharing their experiences with some of the plasmid vectors described in this paper. We also thank Lilly Research Laboratories for supporting this work, and Barbara Fogleman for typing the manuscript.

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