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SHORT PAPER
A vector for studying plasmid stability functions in *Streptomyces*

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Summary

We constructed a cloning vector (pMT603) based on the low copy number plasmid SCP2*. pMT603 is unstable because it lacks the SCP2* stability region and carries the selectable marker thiostrepton-resistance and a tyrosinase gene which results in melanin production. This allows easy testing of plasmid stability and we demonstrated its usefulness by cloning a plasmid stability function.

1. Introduction

The concept of plasmids as non-essential extra-chromosomal genetic elements implies that they can be lost from their host strains. Most plasmids found in nature are very stable and some functions that increase plasmid stability have been identified in *E. coli* plasmids. These include *par* genes that control partition of plasmid copies between daughter cells (Meacock & Cohen, 1980) by an unknown mechanism and site-specific recombination systems that prevent accumulation of plasmid multimers (Summers & Sherratt, 1984).

Streptomyces grow in a mycelial form and, in at least some cases, conjugative plasmids possess 'spread' functions that seem to promote plasmid spread through mycelium (Kieser *et al.* 1982). In the case of the low-copy-number plasmid SCP2* there is a DNA region independent of the conjugation functions which enhances plasmid stability (Bibb, Schottel & Cohen, 1980; Lydiate, Malpartida & Hopwood, 1985). Several genes involved in conjugation, spread, replication and copy number control of SCP2* have been identified by deletion analysis (Larson & Hershberger, 1986), but the stability functions, which lie in another region of the plasmid (Lydiate *et al.* 1985), have not been extensively characterized. A major reason for this is the lack of an easy system to test stability. We have constructed an SCP2*-based vector which allows easy testing of plasmid stability and we illustrate its use to clone a stability fragment.

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2. Materials and Methods

Preparation of plasmid DNA, restriction digests, agarose gel electrophoresis, alkaline phosphatase treatment of DNA, ligation and transformation of *Streptomyces* were as in Kendall & Cullum (1984) and Kendall, Ali-Dunkrah & Cullum (1987). *S. lividans* 66 strain TK64 (*pro*-2 *str*-6) (Hopwood *et al.* 1983) was used as a host for all transformations in this paper. SCP2* DNA was prepared from *S. coelicolor* A3(2) strain M107 (Bibb & Hopwood, 1981) and pIJ702 (Katz, Thompson & Hopwood. 1983) DNA was prepared from a TK64 host strain. R2YE agar medium which was used for propagation of strains and MR2YE agar-containing supplements to enhance melanin production were as in Kendall & Cullum (1984). When necessary, thiostrepton was added to media at a concentration of 50 μg/ml.

(i) *Plasmid stability*

Plasmid stability was scored qualitatively by streaking out spores on MR2YE agar lacking thiostrepton. Unstable strains gave a high proportion of non-melanin-producing colonies (cf. pMT603 and pMT604, Fig. 2). It was possible to test up to eight strains on a single petri dish. For a more quantitative assessment of stability, samples of a spore suspension (about 10^8 spores) produced from a strain grown on thiostrepton-containing medium were spread on R2YE plates and incubated at 30 °C. After sporulation (7–10 days) a spore suspension was harvested and stored for 1 week in glycerol at −20 °C. Dilutions of the spore suspension were plated on MR2YE agar

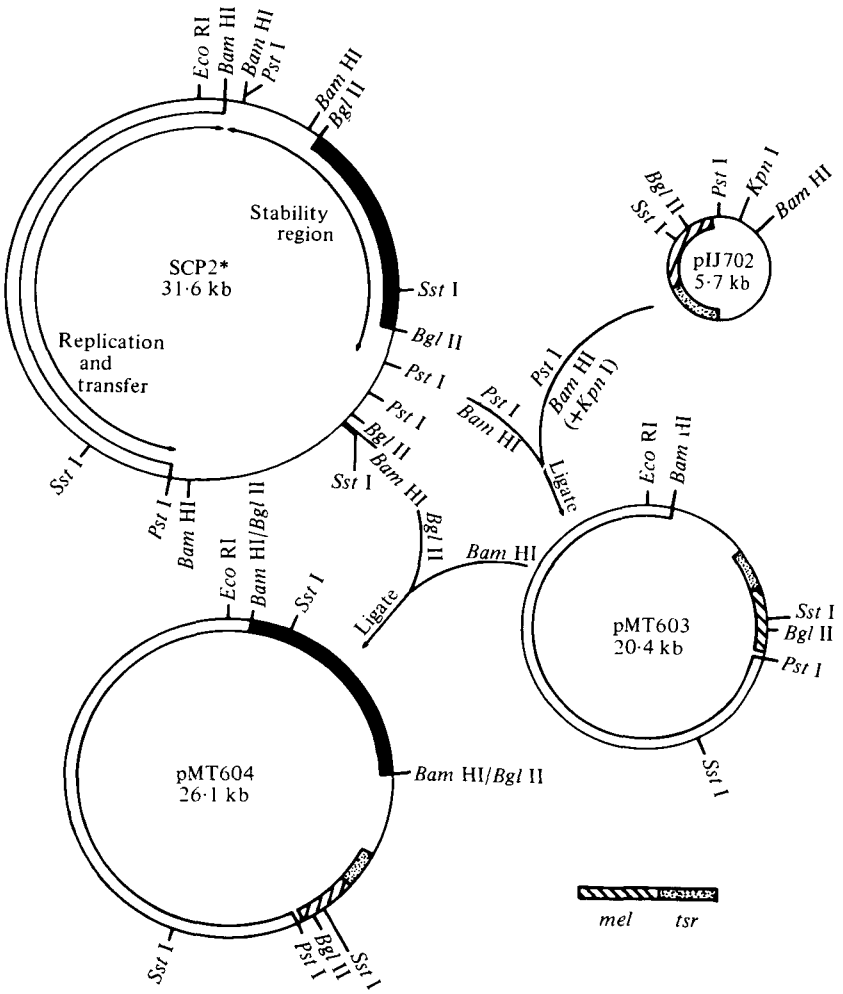

Fig. 1. Construction of pMT603 and pMT604.

and the proportion of melanin-producing colonies counted. After sporulation the colonies were replica-plated on to R2YE-thiostrepton agar to check for thiostrepton resistance.

3. Results

(i) *Construction and characterization of pMT603*

We wanted to clone the tyrosinase (*mel*) gene and the thiostrepton-resistance gene (*tsr*) from pIJ702 into the replication fragment of SCP2*. The 15·5 kb *Bam* HI-*Pst* I fragment of SCP2* (Fig. 1) is known to contain the replication and conjugation functions, but to lack the stability region (Bibb, Schottel & Cohen, 1980). The 5·0 kb *Bam* HI-*Pst* I fragment of pIJ702 carrying the *mel* and *tsr* genes (Fig. 1) does not support replication, because of loss of essential sequences adjacent to the *Bam* HI site (Kieser *et al.* 1982).

Bam HI + *Pst* I double digests of DNA from SCP2* and pIJ702 were ligated together. The pIJ702 DNA was also digested with KpnI and treated with alkaline phosphatase to reduce any background due to intact pIJ702 molecules. The ligation mixture was transformed into *S. lividans* and thiostrepton-resistant transformants were selected. Twenty melanin-producing transformants were tested for stability by restreaking on R2YE agar lacking thiostrepton. Three clones showed considerable instability, producing many Mel$^-$ colonies. Plasmid DNA was prepared from one such clone and restriction analysis with enzymes *Bam* HI, *Bgl* II, *Bcl* I and *Pst* I proved it to have the structure shown in Fig. 1 (pMT603). TK64(pMT603) was grown under thiostrepton selection in liquid culture and yielded about 30 μg CCC-DNA/litre; this is comparable to the yield of other SCP2* derivatives in our laboratory, whereas pIJ702 gives yields over 10-fold higher (i.e. at least 50-fold higher copy number).

(ii) *Cloning of a stability fragment*

pMT603 has unique cloning sites for *Eco* RI, *Bam* HI and *Pst* I which are outside the *mel* region (Fig. 1). We decided to test the usefulness of pMT603 for cloning stability fragments by examining whether we could clone the stability region of SCP2* on a *Bgl* II fragment. DNA from pMT603 was digested with *Bam* HI and treated with alkaline phosphatase. This vector DNA was ligated together with *Bgl* II-digested DNA from SCP2* and introduced by transformation into *S. lividans*. About 1000 thiostrepton-resistant

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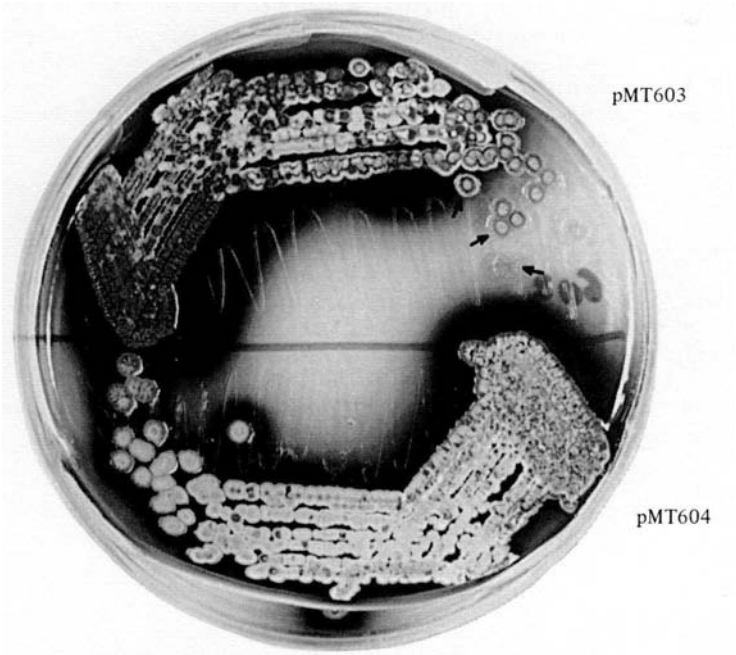

Fig. 2. Comparison of plasmid stability of pMT603 and pMT604 by streaking on non-selective MR2YE agar. Some melanin non-producing (Mel$^-$) colonies of the pMT603-carrying strain are shown by arrows; all of the colonies of the pMT604-carrying strain are Mel$^+$. The host strain for both plasmids is *Streptomyces lividans* 66 strain TK64.

transformants were obtained and 44 of these clones were tested for plasmid stability by streaking out on non-selective medium. Clones that appeared more stable for melanin-production were restreaked twice. Three candidates were identified and plasmid DNA from these was prepared and retransformed into *S. lividans*. Two of the candidates proved to be stable after retransformation and one (pMT604) was selected for further study.

DNA from pMT604 was subjected to restriction analysis with *Bam* HI, *Bgl* II, *Bcl* I and *Sst* I. This showed that pMT604 contained a 5·7 kb *Bgl* II fragment from SCP2* (Fig. 1). The stability of plasmids pMT603 and pMT604 was compared by streaking strains containing them side by side on agar; Fig. 2 confirms that the frequency of plasmid loss is much higher for pMT603 than pMT604. A more-quantitative measure of plasmid stability was the plasmid retention during a spore-to-spore cycle in the absence of selection (see Methods). This showed that pMT604 (180 Mel$^+$/205 total = 85% retention) was much more stable than pMT603 (42/202 = 20% retention). In both cases all Mel$^-$ colonies were also thiostrepton-sensitive.

4. Discussion

We showed that pMT603 can be conveniently used to clone stability genes and that the 5·7 kb *Bgl* II fragment of SCP2* carries such a function. Lydiate *et al.* (1985) deduced that a stability function was present on the partially overlapping 5 kb *Bam* HI–*Sst* I fragment. It should be possible to use pMT603 to localize the stability functions more precisely (e.g by cloning them on smaller Sau3A partial digest fragments). It is impossible to obtain good quantitation of plasmid stability in *Streptomyces* because of the mycelial growth form and the question of the segregation of plasmids into spores; in particular, the number of generations in one spore-to-spore cycle is difficult to estimate and probably depends strongly on medium and growth conditions. However, the *mel* marker on pMT603 allows easy qualitative assessment of stability (Fig. 2).

The instability of pMT603 may be a useful property for experiments in which it is desired to transfer markers from plasmid to chromosome by recombination. Melanin-negative clones that retain markers carried on an insert in pMT603 would be presumed to have transferred the marker into the chromosome. pMT604 may prove a useful cloning vector as it has low copy number and insertions into the *Bgl* II site ought to produce easily recognized melanin-negative colonies as in the high copy number vector pIJ702 (Kate, Thompson & Hopwood, 1983).

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