Temperature-sensitive Mutants of the *Streptomyces* Plasmid pIJ702

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DNA from the *Streptomyces* plasmid pIJ702 was mutagenized *in vitro* using hydroxylamine and transformed into *Streptomyces lividans*. One plasmid with temperature-sensitive replication (pMT660) and one plasmid with a temperature-sensitive tyrosinase (pMT661) were isolated. The plasmid pMT661 contains a novel *Pst*I restriction endonuclease site within the tyrosinase gene.

INTRODUCTION

Temperature-sensitive plasmid replication mutants should be very useful in the study of *Streptomyces*. Such mutants could be used to force replicon fusions and possibly reveal the presence of transposable elements, as has been demonstrated in *Escherichia coli* (Ohtsubo *et al.*, 1980). In addition, they could be used as convenient vectors for the development of a transposon mutagenesis system, the advantages of which have already been well defined in other species (Kleckner *et al.*, 1977). There are also other experiments where easy elimination of plasmid encoded sequences from a cell is desired.

We used the chemical mutagen, hydroxylamine (Humphreys *et al.*, 1978), to produce a temperature-sensitive mutation of plasmid pIJ702 (Katz *et al.*, 1983), which is a widely used cloning vector. The mutagenesis was done *in vitro* on plasmid DNA, the DNA being subsequently introduced into *Streptomyces lividans* by transformation for recovery of mutants. Such a mutagenesis procedure permits high level mutagenesis of a small genome, coupled with easy control of the level of the mutagenic treatment. The plasmid pIJ702 was used because it can be introduced into most *Streptomyces* species; it is a derivative of the broad host range plasmid pIJ101 (Kieser *et al.*, 1982). It carries the easily identified genes for thiostrepton resistance and melanin production, thus allowing easy assay of mutagenesis.

We report here the successful generation of a temperature-sensitive plasmid replication mutant and a temperature-sensitive tyrosinase mutant.

METHODS

Bacterial strains and culture conditions. *S. lividans* strain TK64 (*pro*-2, *str*-6, SLP2⁻, SLP3⁻) (Hopwood *et al.*, 1983) and *S. lividans* strain 3131 containing the plasmid vector pIJ702 (Katz *et al.*, 1983) were kindly supplied by Professor D. A. Hopwood (John Innes Institute, Norwich, UK). They were cultured on the following media: CM (complete medium; Hopwood, 1967), nutrient agar (Oxoid) and R2YE (regeneration yeast extract medium; Chater *et al.*, 1982). The melanin (Mel) phenotype of plasmid pIJ702 was scored by the addition of tyrosine (400 µg ml⁻¹), methionine (100 µg ml⁻¹) and CuSO₄·5H₂O (5 µg ml⁻¹) (Kieser, 1979). All liquid cultures were grown in LB (Luria broth; Maniatis *et al.*, 1982) except for the tyrosinase assay for which glucose/yeast extract medium (Lerch & Ettlinger, 1972) was used. Thiostrepton (E. J. Squibb and Sons, New Brunswick, NJ, USA) was added to liquid media (5 µg ml⁻¹) and solid media (50 µg ml⁻¹). Transformants were selected by overlaying the R2YE regeneration plates with liquid tryptone/salt medium (0.6% (w/v) agar, 16 g tryptone l⁻¹, 10 g yeast extract l⁻¹, 5 g NaCl l⁻¹) containing thiostrepton (500 µg ml⁻¹), 16 h after transformation. Normal (28 °C) and restrictive (39 °C) incubation temperatures were used.

Isolation of genomic DNA. Cells were grown for 4 to 5 d in LB (100 ml), harvested and washed in 10.3% (w/v) sucrose. After resuspension in 5 ml lysozyme solution (10.3% (w/v) sucrose, 25 mM-EDTA, 25 mM-Tris pH 8.0, 2 mg lysozyme ml⁻¹) the cells were incubated for 1 h at 37 °C, and then 0.7 ml protease (10 mg ml⁻¹; Papaya, Sigma) and 1.2 ml 0.5 M-EDTA were added. After incubation for 10 min at 37 °C the cells were lysed by the addition of 1 ml 10% (w/v) SDS and were incubated for 1 h. Sodium chloride was added to a concentration of 0.5 M and protein extracted using 5 ml phenol equilibrated with TE/0.5 M-NaCl/0.01% (w/v) hydroxyquinoline (TE, 10 mM-Tris/1 mM-EDTA pH 8.0). After centrifugation (20 min, 16000 r.p.m., 23600 g) in a Sorvall SS34 rotor, DNA was precipitated from the aqueous phase by the addition of isopropanol. The DNA was recovered by centrifugation (10 min, 10000 r.p.m., 9200 g), resuspended in TE containing 40 µg RNAase ml⁻¹ and incubated for 30 min at 37 °C. The DNA was then subjected to a second phenol extraction and isopropanol precipitation, and was resuspended in 5 ml TE buffer. It was subjected to a further isopropanol precipitation in order to remove all traces of phenol.

Isolation of plasmid DNA. Plasmid DNA was isolated according to the method of Kieser (1984) for small scale work. Large scale preparations were made as follows: cultures in LB (100 ml) containing thiostrepton (5 µg ml⁻¹) were harvested after 3 to 4 d and resuspended in 10 ml buffer (10.3% (w/v) sucrose, 25 mM-EDTA, 25 mM-Tris). The cells were incubated for 30 min at 37 °C after the addition of 1 ml lysozyme solution (50 mM-Tris/HCl pH 8.0 containing 40 mg lysozyme ml⁻¹). Lysis was achieved by adding 20 ml 0.2 M-NaOH/1% (w/v) SDS. After 5 min on ice, protein, high molecular weight RNA and chromosomal DNA were precipitated by the addition of 15 ml 3 M-sodium acetate pH 4.8 and were left on ice for 1 h. The precipitate was pelleted by centrifugation (30 min, 12000 r.p.m., 19200 g). Plasmid DNA was precipitated from the aqueous phase by the addition of ethanol (120 ml) and was left at -20 °C for 1 h. After centrifugation the pellet was resuspended in TE buffer (10 ml), to which was added caesium chloride (10.25 g) and 0.25 ml ethidium bromide (10 mg ml⁻¹). This was centrifuged (60 h, 36000 r.p.m., 90000 g) in a Ti50 rotor in an LS-50B ultracentrifuge (Beckman). DNA bands were visualized using UV light.

Hydroxylamine mutagenesis. Samples of pure plasmid DNA were added to TE buffer to give a final concentration of 2 µg in 60 µl. To this was added 180 µl hydroxylamine solution (hydroxylamine HCl 1.5 M, EDTA 25 mM pH 6.0), 5 µl 0.25 M-EDTA and 13 µl 1 M-Tris/HCl pH 8.0. The reaction mixture was then incubated for 30 min at the required temperature without agitation. DNA was precipitated by the addition of sodium acetate (pH 6.0), to a concentration of 0.4 M, and 800 µl absolute ethanol, and 15 min incubation at -70 °C. The DNA was recovered by centrifugation and resuspended in 20 µl TE and 20 µl 10.3% (w/v) sucrose for transformation into *S. lividans*. Protoplasts were prepared by the method of Chater *et al.* (1982). Transformants were selected as described above.

Restriction endonuclease mapping of plasmids. All restriction enzymes were bought from Boehringer. DNA fragments were separated using horizontal agarose gel electrophoresis in TBE (Tris/borate/EDTA buffer) and agarose concentrations between 0.7% (w/v) and 1.5% (w/v) (Maniatis *et al.*, 1982). Size markers were constructed by digesting phage λ DNA with each of the following restriction endonucleases: *EcoRI*, *HindIII*, *BglI*. The fragment sizes were calculated from the published λ DNA sequence (Sanger *et al.*, 1982).

Determination of enzyme activity. Crude extracts containing the tyrosinase enzyme (EC 1.14.18.1) were prepared from liquid culture using essentially the method described by Katz *et al.* (1983). Induction of the tyrosinase was achieved by the addition of methionine (10 µg ml⁻¹) (Baumann & Kocher, 1976) and CuSO₄ · 5H₂O (5 µg ml⁻¹).

The temperature sensitivity of the tyrosinases isolated by this method was assayed by subjecting samples of the enzyme extracts to incubation for 15 min at various temperatures before measuring their activity at 30 °C. Tyrosinase activity was determined using the dopachrome assay procedure of Lerch & Ettlinger (1972) with L-DOPA (dihydroxyphenylalanine) as substrate and using the conditions of Katz *et al.* (1983). Formation of the pigment dopachrome was followed by measuring the increase in absorbance at 475 nm using a Cecil CE292 digital spectrophotometer (Cambridge, England).

The protein concentrations of the crude extracts were assayed using the Bio-Rad protein assay kit.

RESULTS AND DISCUSSION

Mutagenesis of plasmid pIJ702

Plasmid pIJ702 contains two markers, the thiostrepton resistance gene (*tsr*) and the tyrosinase gene (*mel*). The former gene was used to select for transformants; the latter, whose expression leads to the production of black melanin pigment, was used to assay the efficiency of the hydroxylamine mutagenesis.

Hydroxylamine mutagenesis was known to be strongly temperature dependent (Humphreys *et al.*, 1978), and this is probably because transient DNA strand separation is necessary. Preliminary experiments indicated that an incubation temperature in the range of 75 °C to 85 °C

was capable of producing an acceptable level of both mutagenesis and plasmid survival. The degree of mutagenesis was monitored by scoring the appearance of Mel⁻ colonies. At an incubation temperature of 81 °C, plasmid survival was 1 to 5% and 1% of the thiostrepton resistant colonies were Mel⁻. The background of spontaneously Mel⁻ mutants of pIJ702 was 0.1%, but all spontaneous Mel⁻ mutants had easily detectable deletions (seven of seven tested). DNA from 15 Mel⁻ thiostrepton resistant colonies, found after hydroxylamine mutagenesis, were screened by agarose gel electrophoresis. Only two showed evidence of deletions, the remaining 13 were the same size as pIJ702 and thus probably carried hydroxylamine-induced base changes.

Selection strategy

Hydroxylamine mutagenized pIJ702 DNA was transformed into *S. lividans* TK64 protoplasts and transformants were selected at 28 °C. After sporulation, the transformants were patched to pairs of CM plates which were incubated at 28 °C and 39 °C. The highest temperature which permitted sufficient sporulation for subsequent replica platings was 39 °C. After sporulation, the master plates from each temperature were replica plated to nutrient agar plates containing thiostrepton, and control plates lacking the antibiotic. The replica plates were incubated at the same temperature as their respective master plates. The plates were then scored for possible temperature-sensitive plasmid replication mutants. Such candidates exhibited a simultaneous loss of thiostrepton resistance and ability to produce the black pigment melanin at 39 °C. A total of 800 colonies were screened and eight candidates were isolated. After retesting, these proved to include a temperature-sensitive replication mutant (pMT660) and a temperature-sensitive tyrosinase mutant (pMT661); the other six candidates were indistinguishable from pIJ702.

We have demonstrated the suitability of hydroxylamine treatment *in vitro* for generating plasmid mutants of *Streptomyces*. A high incubation temperature of 81 °C (compared to 60 °C for an *E. coli* plasmid; Humphreys *et al.*, 1978) is necessary probably because of the high G + C content of *Streptomyces* DNA. This mutagenesis system gives reproducible results and high yields of mutants with low lethality. Thus, hydroxylamine is an attractive mutagen for use with *Streptomyces* plasmids.

A temperature-sensitive plasmid replication mutant, pMT660, of pIJ702

S. lividans TK64 containing plasmid pMT660 exhibited a simultaneous loss of thiostrepton resistance and melanin production at 39 °C. Plasmid DNA was isolated from the strain after growth at 28 °C. The temperature-sensitive phenotype was retained on retransformation, locating the lesion to the plasmid. Growth at 39 °C on R2YE for 6 d (R2YE inoculated with 200 µl of a 5 ml spore suspension prepared from a confluent sporulating R2YE + thiostrepton plate at 28 °C) followed by subsequent incubation at 28 °C failed to recover the plasmid determined phenotypes, indicating that the plasmid is lost on incubation at 39 °C. Repeated attempts to isolate plasmid DNA after growth at 39 °C proved unsuccessful. Total DNA was prepared from the strain after growth at 39 °C; it was digested with the enzyme *Bcl*I and subjected to horizontal agarose gel electrophoresis. The DNA was transferred to a Biotrans filter (Pall Ultrafine Filtration Corp., Glen Cove, NY 11542, USA) by the procedure of Southern (1975) and hybridized to ³²P-labelled (Rigby *et al.*, 1977) pIJ702 DNA. No hybridization, in conditions which would reveal even single copy sequences, was seen, demonstrating that growth at 39 °C resulted in the total loss of plasmid DNA sequences from the cell.

The plasmid pMT660 was indistinguishable from pIJ702 when digested with the following enzymes: *Bam*HI, *Bcl*I, *Bgl*II, *Eco*RI, *Hind*III, *Kpn*I, *Pst*I, *Sac*I, *Sac*II, *Sal*GI, *Sma*I, *Xho*I, *Xho*II. pMT660 should prove to be a valuable tool for use in the detection of transposable elements in replicon fusions of *Streptomyces* DNA, and it could be employed as a vehicle in a transposon mutagenesis system. Additionally, pMT660 might be used as an alternative to phage ϕ C31-based vectors used in mutational cloning systems (Chater & Bruton, 1983). We have already used this plasmid to isolate fusions in which the vector has integrated into the host's chromosomal sequences under selection at 39 °C (unpublished results). Investigation of the junction sequences of these fusions is now under way.

Fig. 1. Restriction map of pMT661, a temperature-sensitive tyrosinase mutant of pIJ702.

A temperature-sensitive tyrosinase mutant, pMT661, of pIJ702

S. lividans TK64 containing plasmid pMT661 was thiostrepton resistant, but failed to produce melanin at 39 °C. The ability to produce this pigment was, however, recovered at 28 °C. Plasmid DNA was prepared from the strain grown at 28 °C and 39 °C. The temperature-sensitive phenotype was retained on retransformation, indicating a plasmid location for the lesion. We assayed the temperature-sensitivity of the tyrosinase enzyme by preparing crude enzyme extracts (see Methods) from strains carrying pIJ702, pMT660 and pMT661. The enzyme activity was assayed at 30 °C. Samples of the enzyme were subjected to a 15 min pre-incubation at different temperatures in order to determine the heat-lability of the tyrosinase. Activities were corrected for protein concentrations in the extract and compared to a standard activity (tyrosinase from pIJ702 at 30 °C). These results showed only a slight drop in activity of the enzymes from strains containing pIJ702 and pMT660 over a temperature range of 30 °C to 50 °C. The tyrosinase from pMT661, however had the following activities over this temperature range: 30 °C, 40%; 39 °C, 10%; 45 °C, <0.5%; 50 °C, no detectable activity. This shows that the pMT661-encoded tyrosinase has a marked heat-lability, suggesting that the mutation is indeed in the tyrosinase coding sequence.

Plasmid pMT661 was subjected to a restriction analysis using the enzymes described previously. The vector contained a novel *Pst*I site. This new site was mapped to a point with the co-ordinates 3.85 kb in the pIJ702 map of Katz *et al.* (1983) (Fig. 1) and is within the tyrosinase coding region (M. Bibb, personal communication). The novel *Pst*I site is probably the site of the temperature-sensitive lesion. Only 1% of mutagenized survivors showed a Mel⁻ phenotype; hence, the probability of two independent mutagenic events occurring within the melanin gene is small.

The recovery of this mutant was fortuitous because the initial screening was for loss of thiostrepton resistance. The mutant was probably isolated by mistake due to poor replication to the thiostrepton-containing plates. At present, work is under way to eliminate the original *Pst*I site, at 4.85 kb, from pMT661 in order to construct a cloning vector in which the tyrosinase gene is inactivated by insertion into a single *Pst*I site.

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