Cloning and Expression of the Tyrosinase Gene from *Streptomyces antibioticus* in *Streptomyces lividans*

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In two separate studies a *Bcl*I-generated DNA fragment coding for the enzyme tyrosinase, responsible for melanin synthesis, was cloned from *Streptomyces antibioticus* DNA into two SLP1.2-based plasmid vectors (pIJ37 and pIJ41) to generate the hybrid plasmids, designated pIJ700 and pIJ701, using *S. lividans* 66 as the host. The fragment (1.55 kb) was subcloned into the multicopy plasmid pIJ350 (which carries thiostrepton resistance and has two non-essential *Bcl*I sites) to generate four new plasmids (pIJ702–pIJ705) with the tyrosinase insert located in either orientation at each site. All six plasmids conferred melanin production (the Mel⁺ phenotype) on their host. As in the *S. antibioticus* parent, strains of *S. lividans* carrying the gene specifying tyrosinase synthesis possessed an enzyme activity which was inducible. Most of the tyrosinase activity was secreted during growth of *S. antibioticus*; in contrast, the majority remained intracellular in the *S. lividans* clones. The specific activity of the induced tyrosinase activity (intracellular) was higher (up to 36-fold) when the gene was present on the multicopy vector in comparison with its location on the low copy plasmids, pIJ700 or pIJ701, or in *S. antibioticus*.

Restriction mapping of the tyrosinase fragment in pIJ702 revealed endonuclease cleavage sites for several enzymes, including single sites for *Bgl*II, *Sph*I and *Sst*I that are absent from the parent vector (pIJ350). Insertion of DNA fragments at any one of these sites abolished the Mel⁺ phenotype. The results indicate that pIJ702 is a useful cloning vector with insertional inactivation of the Mel⁺ character as the basis of clone recognition.

INTRODUCTION

The ability to synthesize melanin or melanin-like pigments is a common property among streptomycetes (Küster, 1976); moreover, similar pigments are produced by fungi (Fling *et al.*, 1963; Bouchilloux *et al.*, 1963; Bull & Carter, 1973), unicellular bacteria (Pomerantz & Murthy, 1974), plants (Robb *et al.*, 1965; Vaughan & Butt, 1970), insects (Karlson *et al.*, 1964; Nakamura & Sho, 1964) and mammalian cells (Pomerantz, 1963; Burnett, 1971). Melanin formation from tyrosine is due to the catalytic activity of the enzyme tyrosinase (EC 1.14.18.1), which has been purified to homogeneity from various sources (Fling *et al.*, 1963; Bouchilloux *et al.*, 1963; Vaughan & Butt, 1970; Burnett, 1971; Lerch & Ettlinger, 1972; Bull & Carter, 1973; Pomerantz & Murthy, 1974).

In *Streptomyces*, tyrosinase has interesting genetic determination and regulation. For example, studies with *Streptomyces glaucescens* have revealed that the enzyme is inducible by different amino acids, is synthesized intracellularly and is then secreted into the culture medium (Baumann & Kocher, 1976; Baumann *et al.*, 1976; Kieser *et al.*, 1981). It has been reported that

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the intracellular and extracellular forms of tyrosinase (*S. glaucescens*) are identical in molecular weight, copper content, the sequence of the first 19 amino acids at the *N*-terminal end and the ratio of cresolase to catecholase activity (Crameri *et al.*, 1982). Mutants (structural, regulatory, constitutive, secretion-negative) have been isolated (Baumann & Kocher, 1976; Baumann *et al.*, 1976; Kieser *et al.*, 1981), but little detailed information is available concerning the synthesis and translocation of tyrosinase activity. In many strains melanin production is an unstable property, indicating involvement of a special class of genetic element – either chromosomal as in *S. glaucescens* (Crameri *et al.*, 1982) or possibly plasmid-borne as reported with *S. scabies* (Gregory & Shyu, 1961; Gregory & Huang, 1964). Questions remain regarding the location of the genetic determinants for tyrosinase synthesis and secretion.

Efficient interspecific cloning systems are available for use in *Streptomyces*. Thus, Bibb *et al.* (1980) cloned a methylenomycin resistance gene from *S. coelicolor* DNA and our laboratory has described the cloning of several antibiotic (neomycin, thiostrepton, viomycin, erythromycin) resistance determinants and nutritional genes in SLP1.2 and SCP2 derivatives employing PEG-assisted transformation and transfection in *S. lividans* 66 (Suarez & Chater, 1980; Thompson *et al.* 1980, 1982a).

The experiments reported here describe the cloning of a DNA fragment containing the tyrosinase gene from *S. antibioticus* into the *Streptomyces* plasmid vectors, pIJ37 and pIJ41. Preliminary structural and biochemical characterization of the clones and use of the tyrosinase gene in vector construction are also presented.

METHODS

Media. Most of the media have been described previously; glucose-yeast extract medium (GYM) (Lerch & Ettlinger, 1972); yeast extract-malt extract plus 34% (w/v) sucrose (YEME) (Bibb *et al.*, 1977); R2YE medium (Thompson *et al.*, 1980); P medium (Okanishi *et al.*, 1974) and a modification (MMT) of the minimal medium (MM) described by Hopwood (1967). GYM was employed with D-glucose, 0.4% plus $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.00025%; MMT (200 ml) for tyrosinase expression was minimal medium with 4 ml Difco Casamino acids (30%, w/v); 1.5 ml of a solution containing 1% L-histidine; 0.75% each of L-arginine, L-cystine, L-leucine, L-methionine, L-homoserine, L-phenylalanine, L-proline; 0.15% each of adenine and uracil; 0.01% each of nicotinamide and thiamin, 10 ml of an L-tyrosine solution (7.5 mg ml⁻¹) and 1.5% Bacto agar. Tryptone Soya Broth (TNB) was obtained from Oxoid. T and L media have been described elsewhere (Thompson *et al.*, 1982a).

For the selection of antibiotic-resistant strains, MMT contained neomycin sulphate (Sigma), 1 µg ml⁻¹ or thiostrepton, 50 µg ml⁻¹. Thiostrepton was kindly provided by Mr S. J. Lucania of E. J. Squibb and Sons, New Brunswick, N.J., U.S.A.; a stock solution containing 50 mg ml⁻¹ in dimethylsulphoxide was added to the molten agar medium. Neomycin sulphate solutions were filter sterilized.

Bacterial strains. The recipient organisms used as hosts for recombinant plasmids were the following wild-type strains or mutants derived from them: *S. lividans* 66 (JII strain number 1326); *S. coelicolor* A3(2); *S. griseus* CUB 94; *S. parvulus* ATCC 12434; *S. acrimycini* IPV 1610; *S. albus* G; *S. vinaceus* NCIB 8852 and *S. clavuligerus* ATCC 27064. Donor organisms were *S. antibioticus* IMRU 3720 and *S. griseus* IMRU 3570.

DNA preparations. Total DNA was isolated from mycelium using the procedure described by Chater *et al.* (1982). Large-scale plasmid isolation was by the method of Bibb *et al.* (1977). Plasmid DNA was prepared on a small scale by an alkaline lysis procedure using a modification (Chater *et al.*, 1982) of the method of Birnboim & Doly (1979).

Restriction endonuclease digestions, agarose and polyacrylamide gel electrophoresis. These were carried out as described by Thompson *et al.* (1982b). Restriction endonucleases were purchased from BRL or Boehringer-Mannheim. Reaction mixtures (20 µl) generally contained 10 mM-Tris/HCl, pH 7.4, 10 mM-MgCl₂, 50 mM-NaCl, 1 mM-mercaptoethanol and DNA. Incubations for 4 h at 37 °C (*Bcl*I, 55 °C), were initiated by addition of the restriction endonuclease. Reactions were usually terminated by heating at 70 °C for 10 min; *Bcl*I digestions were terminated by precipitation with ethanol (–20 °C) or by addition of 0.1% (v/v) diethyl pyrocarbonate. Fragment sizes were measured by means of a Houston digitizer (Houston Instruments, Austin, Tex., U.S.A.) connected to an Apple II computer, using *Hind*III fragments of bacteriophage lambda DNA as molecular weight standards (Kieser, 1982).

Preparation and transformation of protoplasts. These were carried out essentially as described by Thompson *et al.* (1982a).

Shotgun cloning experiments. *Bcl*I-digested total DNA of *S. antibioticus* was ligated with *Bcl*I-cleaved pIJ37 or *Bam*HI-digested pIJ41, using standard recombinant DNA techniques (digestions and ligations were monitored by agarose gel electrophoresis). Details of the procedure have been reported elsewhere (Thompson *et al.*, 1982a).

Ligated DNA was used to transform protoplasts ($3-4 \times 10^9$) of *S. lividans* 1326 which were regenerated on R2YE plates and then replicated to MMT containing either neomycin or thiostrepton.

Matings. Spore suspensions of donor *S. lividans* 3129 (pIJ700, Neo^R, Mel⁺) or *S. lividans* 3130 (pIJ701, Thio^R, Mel⁺) and recipient *S. lividans* TK64 (*pro-2*, *str-6*) were mixed on slants of R2YE (Hopwood, 1967). After 6–9 d, spores were scraped from the slant, filtered through cotton plugs and plated under selective conditions on appropriately supplemented MM.

Enzyme assays. *Streptomyces antibioticus* or *S. lividans* was cultivated in GYM liquid (25 ml per 250 ml flask) medium (Lerch & Ettlinger, 1972) at 30 °C on a rotary shaker (model G25, New Brunswick). Induction of tyrosinase activity was by addition of Bacto tryptose (10 mg ml⁻¹, final concentration) and CuSO₄·5H₂O (5 µg ml⁻¹, final concentration) to rapidly-growing cultures of *S. antibioticus* (22–24 h) or *S. lividans* (36–38 h). Mycelium, collected by suction filtration on Whatman no. 2 paper using a Buchner funnel, was washed twice with 25 ml cold sodium phosphate buffer (0.05 M, pH 7.2) and stored at –20 °C prior to use. Mycelium was disrupted in the same buffer by ultrasonic treatment (Dawes soniprobe; Dawes Instruments, London, U.K.) or passage twice through a French pressure cell (Aminco Instrument Co., Urbana, Ill., U.S.A.). After centrifugation at 12000 r.p.m. to remove intact cells and cellular debris, the supernatant was decanted and used as the source of intracellular enzyme. Extracellular tyrosinase activity was examined employing the culture filtrate.

Tyrosinase activity was measured using the dopachrome assay procedure (Lerch & Ettlinger, 1972) with L-dihydroxyphenylalanine (L-DOPA) as substrate. L-DOPA (10 µmol) in sodium phosphate buffer (0.1 M, pH 6.0), additional buffer and enzyme in a total volume of 1.1 ml was incubated at 30 °C; formation of dopachrome was monitored continuously by measuring the increase in absorbance at 475 nm, using a Gilford recording spectrophotometer (Oberlin, Ohio, U.S.A.). One unit of enzyme activity is the amount of enzyme that catalyses the oxidation of 1 µmol L-DOPA per min. The molecular extinction coefficient for the reaction at 475 nm is 3600. Specific activity is the number of enzyme units per mg of protein.

Protein. This was determined by the Lowry method using crystalline bovine serum albumin as standard.

Hybridization studies. ³²P-labelled plasmid DNA or DNA fragments were prepared according to the procedure of Rigby *et al.* (1977). DNA fragments separated by agarose gel electrophoresis (Tris/acetate/EDTA buffer, pH 8.3) were denatured in alkali, neutralized and transferred to nitrocellulose filters, using the Southern blotting technique (Southern, 1975). Filters were preincubated for 6 h at 68 °C in 6 × standard saline citrate (SSC), 1 × Denhardt's solution (Denhardt, 1966) and salmon sperm DNA (50 µg ml⁻¹). Hybridizations were performed in small plastic tubes (4.5 ml). The hybridization mixtures contained 2 × SSC, 1 × Denhardt's solution, 0.1% SDS, 1 mM-EDTA (pH 7.5), 50 µg sheared, denatured salmon sperm DNA ml⁻¹ and about 0.01 µg ³²P-labelled DNA ml⁻¹ (1–5 × 10⁶ c.p.m.). Prior to hybridization, the DNA was denatured by boiling for 10 min. After hybridization at 68 °C for 24 h, the filters were washed 10 times in 2 × SSC, 0.5% SDS at 68 °C over a 50 min period. A final wash was performed in 0.1 × SSC, 0.5% SDS. The filters were dried under a red lamp for 30 min; filters were then exposed to Kodak X-Omat film for autoradiography at –70 °C for 5–30 h.

RESULTS

Cloning of the tyrosinase gene

Simplified restriction endonuclease cleavage maps of the cloning vectors pIJ37 and pIJ41 are shown in Fig. 1. pIJ37 carries the gene specifying neomycin resistance which was used for selection of transformants and has a unique *Bcl*I site which was employed for the initial cloning. pIJ41 contains the determinant specifying thiostrepton resistance (for selection of transformants); the *Bam*HI site within the neomycin phosphotransferase (*aph*) gene was used in a subsequent cloning, making use of the insertional inactivation of neomycin resistance for clone recognition (Thompson *et al.*, 1982*b*).

To clone the tyrosinase gene, *S. antibioticus* DNA was digested with *Bcl*I. DNA fragments were ligated to *Bcl*I-cleaved pIJ37 or to *Bam*HI-digested pIJ41. Ligation mixtures were used to transform protoplasts of the naturally Mel[–] *S. lividans* 1326. After regeneration on R2YE plates, well-sporulated lawns were observed to contain numerous pocks (zones of growth inhibition) due to the Ltz⁺ reaction associated with transfer of SLP1.2-derived plasmids into a plasmid-free host (Bibb *et al.*, 1978). Spores were then replicated on to MMT plates containing tyrosine and either neomycin (pIJ37) or thiostrepton (pIJ41) for selection of drug-resistant transformants, in order to screen for expression of tyrosinase activity. Correspondance between plasmid-encoded phenotypes (Ltz⁺ reaction and patches of drug-resistant growth) indicated approximately 12000 transformants per experiment for each vector (pIJ37 or pIJ41); of these, one clone from each transformation synthesized a black, diffusible pigment, suggesting that it

Fig. 1. Simplified restriction maps of pIJ37 and pIJ41. pIJ37 (14.8 kb) was derived by replacement *in vitro* of a segment of SLP1.2 DNA with DNA carrying the neomycin phosphotransferase (*aph*) determinant of *S. fradiae*; the thiostrepton resistance (*tsr*) determinant (1.0 kb) of *S. azureus* was subsequently cloned into this plasmid to give pIJ41 (15.8 kb) (Thompson *et al.*, 1982b).

had acquired the *Mel*⁺ phenotype. The two *Mel*⁺ clones were purified on selective media and characterized further.

Plasmid DNA (designated pIJ700 and pIJ701, respectively) was isolated from the two putative clones (strains 3129 and 3130) and used to transform protoplasts of *S. lividans*. All the resulting *Ltz*⁺ transformants were also *Mel*⁺ and resistant either to neomycin (pIJ700) or to thiostrepton (pIJ701). Moreover, strains harbouring pIJ701 were sensitive to neomycin. By contrast, insertion of the tyrosinase gene at the *BclI* site (pIJ37 → pIJ700) did not result in a detectable phenotypic change (except *Mel*⁺) in the plasmid vector.

Mating experiments were carried out between *S. lividans* 3129 (pIJ700) or *S. lividans* 3130 (pIJ701), which are streptomycin-sensitive prototrophs, and *S. lividans* TK64 (streptomycin-resistant, proline-requiring). Spores were plated on MMT containing streptomycin and proline and then replica plated to neomycin or thiostrepton. The results confirmed that drug resistance (neomycin or thiostrepton) and the *Mel*⁺ phenotype were transferred to TK64 at high frequency (> 50%).

BclI digestion of pIJ701 yielded two fragments which measured 5.6 kb and >10 kb, respectively. *BclI* + *HindIII* double digests indicated that one cleavage site corresponded to the original *BclI* site of pIJ41 and the other was located about 1.55 kb from the expected position of the cloning site. Ligation of *BclI* fragments into the *BamHI* site of pIJ41 should result in a hybrid *BamHI/BclI* sequence which is not recognized by either enzyme. The finding of a *BclI* cleavage site in the DNA inserted into the *BamHI* site of pIJ41 suggested that two *BclI* fragments had been inserted or that a larger fragment of *S. antibioticus* DNA possessing an internal *BclI* site had been inserted during ligation. Since hybridization studies (see below) showed homology between the DNA inserts of pIJ700 or pIJ701, no further physical analysis of pIJ701 was made.

Restriction endonuclease analysis of pIJ700 was simpler to interpret since the hybrid plasmid had been constructed by insertion of *BclI* fragments into the *BclI* site of pIJ37. *BclI* digestion of pIJ700 resulted in two DNA fragments, one corresponding in size to the single *BclI* fragment of the vector (14.8 kb) and a smaller fragment (1.55 kb) which contained the tyrosinase gene.

Subcloning of the tyrosinase gene into a high copy number plasmid (pIJ350)

pIJ350, derived from the high copy number plasmid pIJ102, was employed for subcloning the tyrosinase gene from the low copy number plasmid, pIJ700. The plasmid vector (4.1 kb) contains three *BclI* sites and carries the gene coding for thiostrepton resistance. pIJ350 is non-conjugative, is present at 40–300 copies per genome, and has a broad host-range (Kieser *et al.*, 1982).

The vector was linearized by partial digestion with *BclI* and ligated with the fragments obtained from *BclI*-cleaved pIJ700 (Fig. 2). After transformation and regeneration of *S. lividans*

Fig. 2. Subcloning of tyrosinase DNA fragment (from pIJ350) into the multicopy number plasmid pIJ700. pIJ350 was partially cleaved with *Bcl*I to generate linear forms (4.1 kb) of the plasmid; pIJ700 was completely digested with the same restriction enzyme, yielding two fragments measuring 1.55 kb and 14.8 kb. Ligation permitted insertion of the 1.55 kb tyrosinase DNA fragment and the construction of six possible hybrid plasmids of which only four types (pIJ702 to 705) could replicate in *S. lividans*. See text for discussion of experiment. The arrow indicates the position of the *Bgl*II site mapped in pIJ702 to pIJ705.

Since pIJ350 contains three *Bcl*I sites, following linearization by partial digestion with *Bcl*I, the tyrosinase DNA fragment could, in principle, be ligated into any one of these sites in either orientation. However, one of the sites is located in a region of the plasmid that is essential for plasmid replication; hence, insertion of foreign DNA into this site leads to the formation of plasmids that cannot be maintained successfully in *S. lividans* (Kieser *et al.*, 1982). Double restriction digests (*Bgl*II + *Pst*I and *Bgl*II + *Cl*aI) were made of plasmid preparations derived from 17 independently-isolated strains. As described below, the tyrosinase DNA insert has a single asymmetric *Bgl*II site and there are single sites for *Pst*I and *Cl*aI in the vector, pIJ350. The sizes of the fragments generated in double digests (*Pst*I + *Bgl*II; *Cl*aI + *Bgl*II) agreed closely with previous data with respect to the location of restriction sites within pIJ350 and the tyrosinase DNA fragment (in pIJ700). Moreover, they showed that the four expected types of hybrid plasmids (Fig. 2) were represented amongst the 17 strains – with type specimens pIJ702 (strain 3131), pIJ703 (strain 3132), pIJ704 (strain 3133) and pIJ705 (strain 3134). The numbers of strains carrying the four types of plasmids were equal (4, 4, 5 and 4, respectively).

Single, double, and partial digests of pIJ702, combined with earlier maps of pIJ101 and its derivatives (Kieser *et al.*, 1982) and the thiostrepton resistance gene (Thompson *et al.*, 1982*b*) enabled us to derive the map shown in Fig. 3. The tyrosinase fragment (1.55 kb) possessed one cleavage site each for *Sma*I, *Sph*I, *Bgl*II, *Sst*II and *Sst*I and three sites for *Sal*GI. There were no recognition sites for *Eco*RI, *Hind*III, *Xba*I, *Pst*I, *Cla*I, *Kpn*I or *Xho*I in the tyrosinase insert. A *Sma*I digest located *Sma*I site 21 relative to *Sma*I site 2. The positions of *Sst*I, *Sal*GI, *Sst*II, *Bgl*II

and *Sph*I sites at positions 15–19 were deduced from double digests using the above enzymes and *Pst*I. Sites 15, 16 and 17 were located very near to one another and had to be ordered by a comparison of the smallest fragments generated by *Bgl*II + *Sst*II, *Bgl*II + *Sal*GI and *Bgl*II + *Sst*I digests on a polyacrylamide gel. The order of the *Sal*GI fragments was determined by a partial *Sal*GI digest.

Insertional inactivation of the Mel⁺ phenotype

The tyrosinase gene can be inactivated by insertion of DNA at the *Bgl*II, *Sst*I or *Sph*I sites (Fig. 4). *Sst*I or *Bgl*II-generated *S. griseus* IMRU 3570 chromosomal fragments were ligated with pIJ702 cleaved with the same enzymes. After transformation and regeneration of protoplasts of *S. lividans*, 10 Mel⁺ and 10 Mel⁻ colonies from each transformation were purified. Mini-lysates derived from all 20 Mel⁺ colonies contained CCC plasmid DNA identical in size to pIJ702; all 20 Mel⁻ colonies yielded CCC DNA larger than pIJ702. After cleavage with *Bgl*II or *Sst*I, inserts of various sizes were observed by agarose gel electrophoresis. Religation and transformation restored the Mel⁺ phenotype. Similarly, an *Sph*I fragment containing the viomycin resistance gene (Thompson *et al.*, 1982a) was used to probe the *Sph*I site in the tyrosinase DNA insert. pIJ702 and pIJ36 (a plasmid containing the viomycin resistance gene cloned into pBR322) were digested with *Sph*I, ligated and used to transform *S. lividans* protoplasts. Viomycin-resistant *S. lividans* transformants were all thiostrepton-resistant and Mel⁻. Cleavage with *Sph*I and religation restored the Mel⁺ phenotype.

Hybridization studies

³²P-labelled pIJ700 and pIJ702 DNA were used as probes in Southern blotting experiments (Southern, 1975) with *Bcl*I-digested DNA from pIJ700, pIJ701 and *S. antibioticus* DNA. These studies confirmed that a *Bcl*I fragment corresponding to the 1.55 kb fragment cloned in pIJ700 is present in *S. antibioticus* DNA. It was also shown (with pIJ702 as the probe) that there is homology between the 1.55 kb fragment from pIJ700 and the larger DNA fragment (> 10 kb) cleaved from pIJ701, indicating identity of the tyrosinase DNA cloned in pIJ700 and pIJ701 (Fig. 5).

Fig. 4. Insertional inactivation of the Mel⁺ phenotype. The tyrosinase gene can be inactivated by insertion of DNA at the *Bgl*II, *Sst*I or *Sph*I sites. DNA fragments cleaved with *Bgl*II were ligated with pIJ702 cleaved with the same enzyme. After transformation and regeneration of protoplasts, spores were plated on MMT containing thiostrepton and L-tyrosine. Colonies developing on the plate were thiostrepton-resistant and exhibited a Mel⁺ or Mel⁻ phenotype.

Fig. 5. Hybridization experiment. pIJ700, pIJ701 and *S. antibioticus* DNA were digested with *Bcl*I and the DNA fragments were separated by agarose gel electrophoresis. After transfer to nitrocellulose filters (Southern, 1975) the DNA fragments were hybridized with nick-translated radiolabelled DNA prepared with pIJ702. The autoradiography revealed a single DNA fragment in *Bcl*I digests of *S. antibioticus* DNA (track 3), and in pIJ700 (track 2), corresponding to the 1.55 kb fragment in *Bcl*I-cleaved pIJ702 (not shown). The hybridizing band in pIJ701 (track 1) indicates that the *Mel* fragments cloned in pIJ700 and pIJ701 are homologous.

Host range of pIJ702

PEG-mediated transformation of pIJ702 into protoplasts of seven other species of *Streptomyces* (*S. coelicolor* A3(2), *S. griseus* CUB 94, *S. parvulus*, *S. albus* G, *S. vinaceus*, *S. acrimycini* and *S. clavuligerus*), with selection for thiostrepton resistance was also observed. In the first six species, transformants were *Mel*⁺. With respect to *S. clavuligerus* transformants were *Mel*⁻, or occasionally weakly *Mel*⁺. However, transformation of *S. lividans* with plasmid DNA from *S. clavuligerus* yielded typical *Mel*⁺ transformants; thus, lack of expression of the *Mel*⁺ phenotype in *S. clavuligerus* was not due to a permanent alteration in the tyrosinase gene.

Biochemical investigations

Strains 3129 to 3134 of *S. lividans* harbouring the recombinant plasmids pIJ700 to pIJ705, respectively, were found to possess an inducible tyrosinase activity (Table 1). Upon addition of

tryptose to GYM medium, there was increased synthesis of the enzyme, although at a slower rate than that observed with *S. antibioticus*. Experiments with the Mel⁺ strains of *S. lividans* revealed that the enzyme was formed intracellularly, but it was not secreted to any significant extent (Fig. 6). The level of activity of the intracellular tyrosinase (induced cells) appeared to be the same in *S. antibioticus* and in strain 3130 of *S. lividans* carrying the low-copy plasmid, pIJ701. There was a higher level of enzyme activity in the strains (induced with tryptose) with the high-copy plasmids (2.5- to 36-fold greater). Additional investigations relating to expression of the tyrosinase gene are currently under investigation.

Table 1. Expression of tyrosinase activity (intracellular) in *S. antibioticus* and in *S. lividans* strains harbouring recombinant plasmids

Organism	Plasmid	Tyrosinase specific activity [units (mg protein) ⁻¹]		Ratio of induced/ uninduced	Relative activity	
		Uninduced	Induced		Uninduced	Induced
<i>S. antibioticus</i>	—	0.015	0.462	30.8	1.0	1.0
<i>S. lividans</i> 3129	pIJ700	0.008	0.184	23.0	0.5	0.4
<i>S. lividans</i> 3130	pIJ701	0.064	0.466	7.3	4.3	1.0
<i>S. lividans</i> 3131	pIJ702	0.286	2.368	8.3	19	5.2
<i>S. lividans</i> 3132	pIJ703	1.803	16.600	9.2	120	36.1
<i>S. lividans</i> 3133	pIJ704	0.636	3.812	6.0	42	8.3
<i>S. lividans</i> 3134	pIJ705	0.129	1.194	9.3	8.6	2.6

Fig. 6. Synthesis of tyrosinase activity of *S. antibioticus* and clones of *S. lividans* (harbouring the plasmid pIJ700, pIJ702 or pIJ703) in GYM medium with or without tryptose (10 mg ml⁻¹). Tryptose was added to 20 h cultures of *S. antibioticus* and to 36 h cultures of *S. lividans*. Mycelium was harvested at 0, 2, 6, and 12 h post-induction with tryptose. Intracellular tyrosinase activity (▲, △) is expressed as units (mg protein)⁻¹; extracellular tyrosinase activity (●, ○) is expressed as units ml⁻¹. Solid line, tryptose present; broken line, tryptose absent.

DISCUSSION

It is likely that the 1.55 kb *Bcl*I fragment of *S. antibioticus* DNA carries natural initiation signals for transcription and translation of the tyrosinase gene since enzyme activity was well expressed, not only in the *Bcl*I site in the primary clone (pIJ700) but also, in both orientations, in the two available *Bcl*I sites of pIJ350. Moreover, tyrosinase activity was inducible in strains carrying any of the six hybrid plasmids, as in the *S. antibioticus* parent, suggesting that regulatory sequences had been cloned together with the structural gene. However, in contrast to the translocation of the enzyme in *S. antibioticus*, tyrosinase remained largely intracellular in the cloned strains. It is possible that a mechanism(s) required for cleavage of a tyrosinase 'signal sequence' (Davis & Tai, 1980) or for post-translational modification of the intracellular enzyme for secretion (Lai *et al.*, 1981; Nielsen *et al.*, 1981; Nielsen & Lampen, 1982) is absent in *S. lividans*. In an analogous study Robbins *et al.* (1981), using as heterologous host *Escherichia coli*, cloned the structural gene for endoglycosidase H from *S. pilicatus* DNA. The intracellular form of the endoglycosidase synthesized by *E. coli* was reported to have a molecular weight of approximately 30000, which contrasts with the secreted enzyme (27000 mol. wt) made by *S. pilicatus*. Further investigations employing both *S. antibioticus* and the cloned strains of *S. lividans* should prove useful for elucidating the control mechanism of tyrosinase synthesis and its secretion.

The *S. antibioticus* gene coding for tyrosinase is a useful component of cloning vectors derived from pIJ101. The cloned *Bcl*I fragment has single *Bgl*II, *Sph*I and *Sst*I sites; thus, insertion of foreign DNA into one of these sites results in insertional inactivation of the tyrosinase gene. As a consequence, white instead of brown or black host colonies develop. The pIJ702 plasmid (at the *Bgl*II or *Sst*I sites) has already proved useful in the isolation of clones carrying components of the glycerol utilization (*gyt*) operon (E. T. Seno, personal communication) and of the undecylprodigiosin gene cluster (J. S. Feitelson, personal communication) of *S. coelicolor*. Further, the presence of the tyrosinase promoter might permit expression of genes (cloned into the *Bgl*II, *Sst*I or *Sph*I sites) that lack a functional promoter sequence. Whether the potential exportability of the tyrosinase system can be utilized in order to achieve secretion of products of cloned genes remains to be established.

Amplification of some products of cloned genes is one of the potential advantages of a high-copy number plasmid, and this appears to be the case for tyrosinase activity. Thus, strains of *S. lividans* harbouring a high-copy number plasmid carrying the tyrosinase gene (pIJ702 to pIJ705) have higher tyrosinase activity (2.5- to 36-fold) than those carrying a low-copy number plasmid (pIJ700, pIJ701) or *S. antibioticus* itself. However, further work will be needed to establish the reason(s) why a strict dosage effect on the level of tyrosinase activity was not observed in strains carrying pIJ702 to pIJ705. Since the site of insertion and/or the orientation of the tyrosinase DNA fragment in pIJ350 greatly influence gene expression, proximity to a potent plasmid promoter or terminator may be important. However, physiological or regulatory factors, such as growth rate, medium pH, availability and utilization of a substrate (or inducer), amount of repressor formed, etc., may also have an effect. It will be of interest to transform Mel⁺ and Mel⁻ strains of *S. antibioticus* with the recombinant plasmids to investigate the influence of both chromosomal- and plasmid-specified determinants upon enzyme synthesis.

Melanin production is a property of sporadic occurrence in many prokaryotes and eukaryotes. The cloned tyrosinase gene of *S. antibioticus*, therefore, may be useful to investigate questions concerning possible evolutionary divergence or convergence of the corresponding gene and enzyme in other organisms. For example, when used as a DNA probe in Southern transfer experiments, it has allowed the recognition of closely-related DNA in other *Streptomyces* species, but not in Mel⁻ strains (H. Schrempf, E. Katz and D. A. Hopwood, unpublished results). It will, therefore, be useful in elucidating the nature of the genetic instability involving melanin production which has been observed in several of the *Streptomyces* species containing related sequences. Gregory & Shyu (1961) and Gregory & Huang (1964) postulated plasmid determination of the Mel⁺ phenotype. Recent evidence suggests that the genes for synthesis and secretion of tyrosinase activity are chromosomal in *S. glaucescens* (Crameri *et al.*, 1982). The cloned tyrosinase gene (*S. antibioticus*) has been also used to establish that the frequent Mel⁻

variants of *S. reticuli* arise after deletion of DNA, including the tyrosinase gene, from the chromosome (H. Schrempf, unpublished results). It is also of interest to note that the first reaction in the biosynthesis of the propylhygric acid moiety of the antibiotic lincomycin is the conversion of L-tyrosine to DOPA (Witz *et al.*, 1971; Michalik *et al.*, 1975). The similarity of this enzyme-catalysed reaction to the one carried out by tyrosinase suggests that the cloning of the relevant gene from *S. lincolnensis* DNA would be readily obtainable. Further, isolation of the eukaryotic gene(s) specifying tyrosinase synthesis (Fling *et al.*, 1963; Pomerantz, 1963; Ottolenghi-Nightingale, 1969; Burnett, 1971) may also be feasible, using similar selection procedures.

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