

Construction and Application of a Promoter-Probe Plasmid That Allows Chromogenic Identification in *Streptomyces lividans*

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We cloned a *Streptomyces coelicolor* A3(2) DNA fragment which directed synthesis of a brown pigment, presumably a shunt product in the actinorhodin biosynthetic pathway, on the plasmid vector pIJ41 in *Streptomyces lividans*. The pigment production was observed only when the DNA fragment was inserted downstream from a functional promoter sequence. By subcloning the fragment together with *in vitro* manipulation, a promoter-probe plasmid vector (pARC1) with a unique *Bam*HI cloning site was constructed that allows chromogenic identification of transcriptional control signals in *Streptomyces lividans* based on the expression of the cloned pigment gene(s). The *Escherichia coli* *tac* (*trp-lac* hybrid) promoter, consisting of 92 base pairs and a promoter region including the leader sequence of erythromycin resistance gene (*ermC*) on staphylococcal plasmid pE194, when ligated in the correct orientation in the *Bam*HI site of pARC1, promoted expression of the cloned pigment gene(s) in *Streptomyces lividans*, whereas the *Saccharomyces cerevisiae* *GAL7* promoter did not. In the case of the *ermC*, induction of the pigment production by the addition of either erythromycin or lincomycin, but not virginiamycin, was observed. The system was also shown to be useful and convenient in isolating transcriptional control signals of *Streptomyces* chromosomal DNA and estimating their activities.

Streptomyces produce the great majority of naturally produced antibiotics as well as various secondary metabolites. These organisms are gram-positive bacteria with an extremely high guanine-plus-cytosine content (70 to 73%) and are the most important group of industrial microorganisms. A complex process of morphological differentiation displayed by *Streptomyces* spp. also has biologically interesting aspects.

The recent development of recombinant DNA technology and molecular cloning in *Streptomyces* spp. has enabled a detailed study of gene expression involving antibiotic production and cell differentiation (1, 4, 6, 7, 9, 11, 28, 37). The nucleotide sequence of the neomycin phosphotransferase gene from *Streptomyces fradiae* (35) revealed that promoter "consensus" sequences (-35 and -10 regions) found in other bacteria (30) were absent, which probably explains why *Streptomyces* genes are not functionally expressed in *Escherichia coli* (2, 20, 32). However, by using a promoter-probe vector with a structural gene of an antibiotic resistance determinant, Bibb and Cohen (2) showed that promoters from both gram-positive and gram-negative bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Serratia marcescens*, and *E. coli* were found to promote expression of a chloramphenicol acetyltransferase gene in *Streptomyces lividans*. Jaurin and Cohen (25) reported that *Streptomyces lividans* RNA polymerase can recognize *E. coli* transcriptional signals including initiation and termination in a similar manner just as *E. coli* RNA polymerase does.

To elucidate *Streptomyces* promoter signals in combination with heterologous forms of *Streptomyces* RNA polymerase, it is important to isolate and characterize various promoter regions capable of regulating gene expression in *Streptomyces* spp. In addition, the isolation of strong promoters would be useful for constructing recombinant plas-

mids that lead to higher expression of cloned genes such as antibiotic biosynthetic genes.

In this paper, we report construction and application of a promoter-probe vector in *Streptomyces lividans*. The plasmid vector, presumably containing a part of the actinorhodin biosynthetic pathway, allowed the isolation of promoters by detection of an experimentally convenient phenotype of pigment production. Furthermore, promoter strengths can be easily determined by estimating yields of the pigment production.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Streptomyces lividans* TK21 (17) and *Streptomyces coelicolor* A3(2) M130 (3) were obtained from D. A. Hopwood. *Streptomyces griseus* FT-1 and *Streptomyces lividans* HH21 were previously described (18). Plasmid pIJ41, which confers resistances to neomycin and thiostrepton, was from D. A. Hopwood (36). Plasmid pDR540 containing the *tac* promoter (31) was provided by K. Yoda. pSR203, as a source of *ermC* promoter, derived from pHW1 (23), was obtained from M. Tsuchiya. Plasmid pSGPC3 containing the *Saccharomyces cerevisiae* *GAL7* gene (29) was from T. Yamashita. Growth media for *Streptomyces* spp. were previously described (18).

Chemicals and enzymes. The restriction endonucleases, T4 DNA ligase, S1 nuclease, and BAL 31 nuclease were purchased from Takara Shuzo, Co., Ltd., or New England BioLabs, Inc. Erythromycin and lincomycin were purchased from Sigma Chemical Co. Virginiamycin (identical to ostreogrycin A; a streptogramin type B antibiotic) and thiostrepton were supplied by Ajinomoto Co., Inc., and Asahi Chemical Industry, respectively.

Recombinant DNA studies. Plasmid and chromosomal DNAs were prepared by the lysozyme-sodium dodecyl sulfate-EDTA method of Horinouchi et al. (20), followed by cesium chloride-ethidium bromide density gradient centrifuga-

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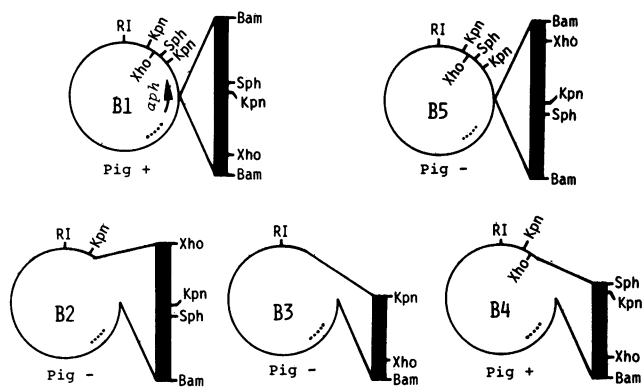


FIG. 1. Schematic summary of plasmid constructions and pigment productivity of constructed plasmids. These include pIJ41-B1, the originally isolated plasmid, capable of causing pigment production (Pig +) in *Streptomyces lividans*; pIJ41-B5, obtained by cloning the same 6.7-kb fragment in the same *Bam*HI site of pIJ41 in the opposite orientation to pIJ41-B1, resulting in a plasmid which has no ability to cause pigment production (Pig -); pIJ41-B2, obtained by insertion of a *Bam*HI-*Xho*I fragment into pIJ41 digested with *Bam*HI plus *Xho*I, resulting in a plasmid incapable of conferring pigment production in *Streptomyces lividans*; pIJ41-B3, obtained by insertion of a *Bam*HI-*Kpn*I fragment into pIJ41 digested with *Bam*HI plus *Kpn*I, resulting in a plasmid with no ability to cause pigment production; and pIJ41-B4, obtained by deletion of a small segment by digestion of pIJ41-B1 with *Sph*I, resulting in a plasmid that causes *Streptomyces lividans* to produce a brown pigment. The *Bam*HI site of pIJ41 is located in the neomycin phosphotransferase (*aph*) gene. The *aph* gene is transcribed in the direction indicated by the arrow (35). Dots stand for the extent of thiostrepton resistance gene. Restriction endonuclease abbreviations are: *Bam*, *Bam*HI, *Kpn*, *Kpn*I; *RI*, *Eco*RI; *Sph*, *Sph*I; and *Xho*, *Xho*I.

gation. General methods including protoplast transformation (37), ethidium bromide-agarose gel electrophoresis (20), and Southern blot DNA-DNA hybridization (19, 33) were previously described.

Yield determination of pigment production by transformants. About 10^8 spores were inoculated in 10 ml of YMPG medium (containing [grams per liter] yeast extract, 4; malt extract, 10; $MgCl_2 \cdot 6H_2O$, 2; Bacto-Peptone, 1; and glucose, 10; pH 7.2) containing 40 μ g of thiostrepton per ml and incubated on a reciprocal shaker at 30°C for 7 days. Acetone (10 ml) was added to the broth, followed by centrifugation to obtain supernatants. Values of optical density at 400 nm (OD_{400}) were measured by using a similarly prepared supernatant from *Streptomyces lividans* carrying pARC1 as the reference with a Jasco UVIDEC-610 scanning spectrophotometer, because preliminary data showed the pigment had a peak at 400 nm.

RESULTS

Cloning of DNA fragment directing pigment production.

Chromosomal DNA of *Streptomyces coelicolor* A3(2) M130 and pIJ41 DNA were digested with *Bam*HI, ligated with T4 DNA ligase, and introduced by transformation into *Streptomyces lividans* HH21. After protoplast regeneration on R2YE medium, thiostrepton-resistant transformants were selected on Bennett agar medium containing 40 μ g of thiostrepton per ml. Among ca. 4,000 transformants, we detected a colony that produced a thick brown pigment on a selection plate. The isolate was neomycin sensitive, indicating that it possessed a recombinant plasmid containing an

insertion sequence at the *Bam*HI site of the vector. Plasmid DNA from this isolate contained a 6.7-kilobase (kb) fragment in the *Bam*HI site as shown by agarose gel electrophoresis. Figure 1 shows the restriction map of this plasmid, named pIJ41-B1. Purified pIJ41-B1 DNA was reintroduced by transformation into *Streptomyces lividans* strains HH21 and TK21, where it directed synthesis of brown pigment. *Streptomyces lividans* TK21, a wild-type strain, did not produce the brown pigment under the conditions examined.

To confirm that the cloned fragment was derived from *Streptomyces coelicolor* A3(2) M130, we performed Southern blot DNA-DNA hybridization by using ^{32}P -labeled, 6.7-kb fragment as probe and *Bam*HI-digested total DNA of *Streptomyces coelicolor* A3(2) M130 as target. A 6.7-kb band was detected (data not shown). In addition, *Streptomyces lividans* TK21 was also found to contain a 6.7-kb *Bam*HI fragment which hybridized to the probe. By comparison of the intensities of the hybridized bands of the two strains, it was suggested that the two strains contained sequences considerably homologous to each other.

Preliminary characterization of the brown pigment. The brown pigment was extracted from a 6-liter culture of *Streptomyces lividans* carrying pARC13 (described below) by column chromatography and high-pressure liquid chromatography. Data from 1H -NMR and ^{13}C -NMR spectra, in which most of chemical shifts characteristic to an oxidized naphthalene (12, 34) were observed, suggested that the pigment consisted of 16 carbons and had an oxidized naphthalene skeleton with a β -hydroxy- δ -keto acid as a side chain. From UV spectra which were pH dependent, it was suggested that the pigment had a naphthoquinone moiety. This

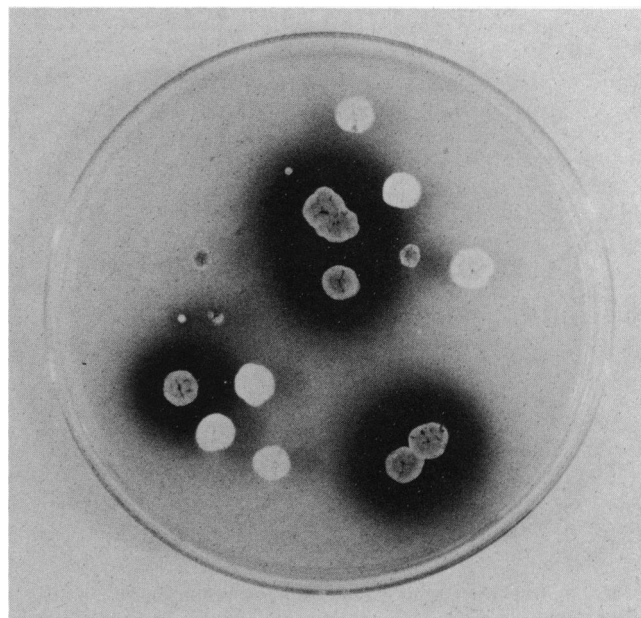


FIG. 2. Phenotypes of pigment-producing and nonproducing colonies. The cloned 6.7-kb fragment purified by agarose gel electrophoresis (8) was ligated with *Bam*HI plus alkaline phosphatase-treated pIJ41 and introduced into protoplasts of *Streptomyces lividans* TK21. Regenerated spores were replicated to Bennett agar medium containing 40 μ g of thiostrepton per ml, in which after 4 days of growth at 30°C, ca. one-third of the transformants showed a pigment-producing phenotype (because of carrying pIJ41-B1) and the other (containing either pIJ41 circularized in vivo or pIJ41-B5) produced no pigment, as shown.

structure is supposedly formed from 8 acetate units via a polyketide, which suggests the pigment is an intermediate or a shunt product in the actinorhodin biosynthetic pathway. Actinorhodin is a diffusible pigment produced by *Streptomyces coelicolor* A3(2) and a pH indicator; it is blue at alkaline pH and red at acidic pH. In fact, the UV and NMR spectra were considerably similar to those of nanaomycin (34) which has a structure considerably similar to that of actinorhodin.

Trimming the cloned DNA fragment by subcloning. Based on the restriction map of pIJ41-B1, we constructed a set of recombinant plasmids (Fig. 1). Plasmid pIJ41-B5, in which the same 6.7-kb fragment was inserted at the *Bam*HI site of pIJ41 in the opposite orientation from that of pIJ41-B1 (Fig. 1), failed to confer pigment production to *Streptomyces lividans* TK21. This result suggested that the cloned gene(s) causing the *Streptomyces lividans* host to produce the brown pigment lacked its own promoter. Taking into consideration the fact that the *Bam*HI site is located in the neomycin phosphotransferase (*aph*) gene and its transcription occurs in the direction indicated in Fig. 1 (35), the orientation of the cloned gene(s) should be from bottom to top in Fig. 1.

To determine the extent of the gene(s), we used pIJ41-B1 as the starting material and deleted a *Kpn*I to *Bam*HI fragment or a *Sph*I to *Bam*HI fragment, resulting in pIJ41-B3 or pIJ41-B4, respectively. Plasmid pIJ41-B4 conferred pigment production to the host cell, whereas pIJ41-B3 did not (Fig. 2). Plasmid pIJ41-B2, which had the same orientation

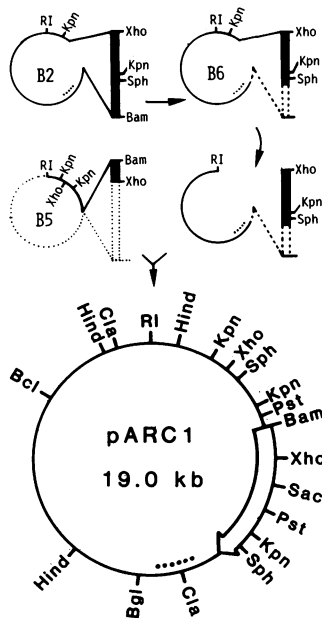


FIG. 3. Construction of promoter-probe vector plasmid pARC1. Abbreviations are the same as described in the legend to Fig. 1. Linear pIJ41-B2 DNA obtained by cleavage with *Bam*HI was digested with BAL 31 nuclease by incubation at 20°C for 3 min. After phenol extraction, the digest was treated with T4 DNA ligase and introduced into *Streptomyces lividans*. A plasmid, named pIJ41-B6, was detected in this way and was found to have a 2.3-kb deletion covering the *Bam*HI site. A linear fragment was purified after digestion of pIJ41-B5 with *Eco*RI completely and *Xho*I partially, as indicated, and ligated with a linear fragment obtained by digestion of pIJ41-B6 with *Eco*RI plus *Xho*I, resulting in a plasmid, named pARC1, which has a unique *Bam*HI site.

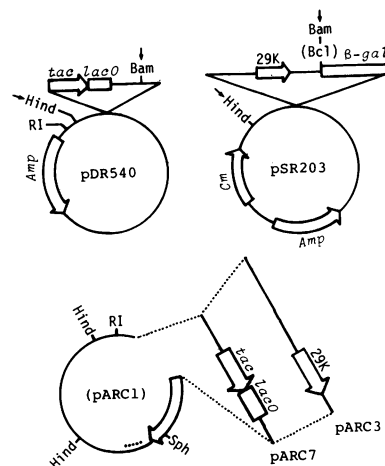


FIG. 4. Schematic representation of plasmids used as sources of the *tac* and *ermC* promoters. The *tac* promoter consisting of 92 bp was excised by digestion of pDR540 with *Hind*III plus *Bam*HI, as indicated by arrows. The 798-bp *ermC* promoter region including the control leader sequence whose nucleotide sequence is derived from pHW1 was isolated from pSR203 in a similar way. The excised *tac* and *ermC* promoters were inserted into pARC1 digested with *Hind*III plus *Bam*HI, resulting in plasmids pARC7 and pARC3, respectively.

as pIJ41-B5, did not cause pigment production. These results indicated that the *Kpn*I site was located in an essential region and that the *Bam*HI to *Sph*I fragment (4.3 kb) carried by pIJ41-B4 was necessary for causing the pigment production.

Construction of promoter-probe plasmid vector pARC1. To confirm that the presence of a transcriptional signal upstream from the *Bam*HI site of the trimmed 4.3-kb fragment leads to pigment production, and to construct a more convenient promoter-probe plasmid vector to manipulate, we deleted unnecessary regions covering one of the *Bam*HI sites located downstream from the cloned gene(s) (Fig. 3). The plasmid pARC1 constructed in this manner consisted of 19.0 kb and contained a unique *Bam*HI cloning site. *Bam*HI produces sticky ends GATC and therefore *Bcl*II-, *Bgl*II-, or *Sau*3AI-created ends can be readily ligated.

Insertion of *tac*, *ermC*, and *GAL7* promoters into the promoter-probe vector. (i) *tac* promoter. The strong consensus *tac* (or *trp-lac* hybrid) promoter in *E. coli* consists of the -35 region of the *trp* promoter, the -10 region of the *lac* UV-5 promoter, and a synthetic ribosome-binding site (31). The 92-base-pair (*bp*) *tac* promoter can be excised by double digestion of pDR540 with *Hind*III plus *Bam*HI (Fig. 4). To insert the *tac* promoter sequence with a *Bam*HI-created sticky end at one end and a *Hind*III-created sticky end at the other end, we digested pARC1 DNA with *Bam*HI completely and *Hind*III partially, after which the largest linear molecule (17.6 kb) with *Bam*HI- and *Hind*III-created sticky ends at both ends were purified by agarose gel electrophoresis. The linear molecules were ligated with the 92-bp *tac* promoter, and the ligation mixture was introduced by transformation into *Streptomyces lividans* TK21. Almost all of the thiostrepton-resistant transformants obtained in this way produced the brown pigment. Plasmid DNA was purified from these pigment-producing colonies and analyzed by agarose gel electrophoresis. Digestion of the plasmid DNA, named pARC7, with *Bam*HI plus *Hind*III gave a 92-bp fragment in addition to the expected three bands, indicating

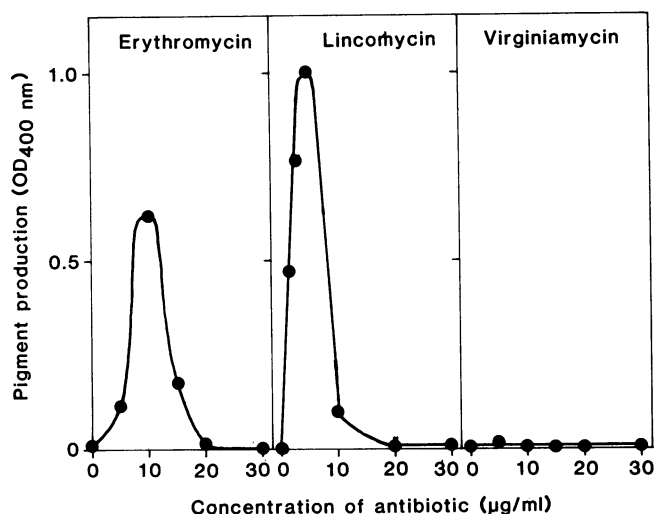


FIG. 5. Induction of pigment production by MLS antibiotics in the *ermC*-pigment gene(s) fusion construction. *Streptomyces lividans* (pARC3) was grown in YMPG liquid medium containing 40 µg of thiostrepton per ml for 3 days as the seed culture. A portion (0.1 ml) of the culture was inoculated in 10 ml of the same medium containing various concentrations of erythromycin, lincomycin, or virginiamycin and incubated at 30°C for 7 days. Pigment production was expressed as the optical density as described in the text, with a *Streptomyces lividans* (pARC3) culture grown in the absence of MLS antibiotics as the reference. Under the cultural conditions examined, *Streptomyces lividans* (pARC3) did not produce a detectable amount of the pigment.

that the *tac* promoter was connected in the correct orientation to the pigment production gene(s) on pARC1.

In another experiment in which a portion of the largest *Bam*HI-*Hind*III linear molecules (17.6 kb) was recircularized by successive treatments with S1 nuclease and T4 DNA ligase, none of the thiostrepton-resistant transformants produced the pigment. This result excludes the possibility that the deletion of the *Hind*III-*Bam*HI sequence (i.e., from 1 o'clock to about 3 o'clock in the circular map of pARC1) increased read-through transcription, if any, from the vector sequence to such an extent that pigment production can be detected.

These results suggest that the *tac* promoter signals are functionally recognized in *Streptomyces lividans*, in accordance with the results of Bibb and Cohen (2). *Streptomyces lividans* carrying pARC7 began to produce a detectable amount of the pigment at a very early stage of growth and appeared to continue the production both on solid medium and in liquid culture. This implies that the *tac* promoter is expressed throughout the vegetative growth.

(ii) *ermC* promoter. The *ermC* gene carried by staphylococcal plasmid pE194 specifies resistances to macrolide, lincosamide, and streptogramin type B (MLS) antibiotics through specific *N*⁶,*N*⁷-dimethylation of adenine in 23S rRNA (27). Expression of the *ermC* gene is regulated by mechanisms of secondary structure rearrangement of the 5' end (leader sequence) of the mRNA leading to methylase synthesis (13, 21). By using gene fusion techniques, Kirsch and Lai (26) and Gryczan et al. (14) reported that the *ermC* promoter including the leader sequence induced the expression of β-galactosidase gene fused downstream from it in *E. coli* and in *B. subtilis*, respectively, when erythromycin at a sublethal concentration was added to the cultures.

To determine whether the *ermC* promoter including the

control leader sequence can promote expression of the cloned pigment gene(s) in such a manner that the addition of MLS antibiotics increases the yield of pigment production, we inserted a promoter region of the *ermC* gene derived by digesting pSR203 with *Bam*HI plus *Hind*III into pARC1 digested with *Bam*HI plus *Hind*III (Fig. 4). The excised *ermC* fragment contains its transcriptional control signals and amino-terminal 113 amino acids of adenine methylase as well as a small segment derived from pC194 sequence (24). The lengths of the mRNA to the AUG start codon of the control leader peptide and the adenine methylase are 33 and 142 bp, respectively (22). Thiostrepton-resistant transformants obtained in this way were found to possess a plasmid, named pARC3, as expected, and to produce a relatively small amount of the brown pigment after 5 days of growth on Bennett agar medium.

Induction specificity was examined in liquid culture, in which pigment production was followed as a function of concentration of MLS antibiotics (Fig. 5). Under the liquid culture conditions tested, pigment production was not observed in *Streptomyces lividans* (pARC3) without the addition of a sublethal concentration of erythromycin or lincomycin. On the solid medium, *Streptomyces lividans* (pARC3) produced a detectable amount of pigment after 5 days of growth in the absence of any MLS antibiotic. In the original host *Staphylococcus aureus* and in *B. subtilis*, only erythromycin and oleandomycin can activate the *ermC* promoter, leading to expression of adenine methylase. The *ermC* promoter region fused to the pigment gene(s) was induced by both erythromycin and lincomycin at a sublethal

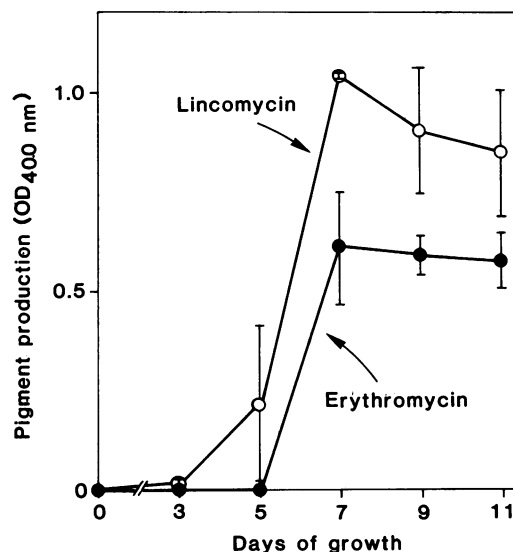


FIG. 6. Time course of pigment production of *Streptomyces lividans* carrying pARC3 in the presence of sublethal concentrations of erythromycin and lincomycin. *Streptomyces lividans* (pARC3) was grown in YMPG liquid medium containing 40 µg of thiostrepton per ml at 30°C for 3 days. A portion (0.1 ml) was transferred to 10 ml of the same medium containing either 10 µg of erythromycin or 5 µg of lincomycin per ml. Pigment production was measured by using cultures of *Streptomyces lividans* (pARC3) grown in a similar way in the absence of any MLS antibiotic as the reference, and is shown as single vertical bars covering the range of values obtained from two independent experiments. *Streptomyces lividans* (pARC3) did not produce a detectable amount of the pigment under the conditions used, but cell pellets obtained by centrifugation were slightly brown after 7 days of growth.

concentration in *Streptomyces lividans*. Virginiamycin, a streptogramin type B antibiotic, did not act as an effective inducer. Figure 6 shows a time course of pigment production in the presence of erythromycin and lincomycin at the optimum concentration for induction. Lincomycin induced the pigment production more effectively than erythromycin.

(iii) **GAL7 promoter.** The *GAL7* gene of *Saccharomyces cerevisiae* encoding galactose-1-phosphate uridylyl transferase is under a complex control involving both *GAL4* and *GAL80* genes in yeasts (16). The nucleotide sequence of the transcriptional initiation signals of *GAL7* was determined by Nogi and Fukasawa (29). A 610-bp fragment (nucleotide number -546 [*Nco*I site] to +65 [*Hinf*I site] of their numbering) capable of promoting expression of the *GAL7* gene in yeasts was excised by digestion with *Bam*HI from pBGPC3 in which both ends of the 610-bp fragment *Bam*HI linkers are attached (T. Yamashita and T. Beppu, unpublished data). The excised *GAL7* promoter region was inserted in two different orientations, whose structures were confirmed by restriction mapping, in the dephosphorylated *Bam*HI site of pARC1. However, none of the transformants showed pigment production, indicating that the *GAL7* promoter was not functionally expressed to such an extent that it allowed chromogenic identification.

Shotgun cloning of chromosomal DNA fragments containing transcriptional control signals. Chromosomal DNA prepared from *Streptomyces griseus* FT-1 and *S. lividans* TK21 was digested with *Sau*3AI, ligated into the dephosphorylated *Bam*HI cleavage end of pARC1 through the common four-

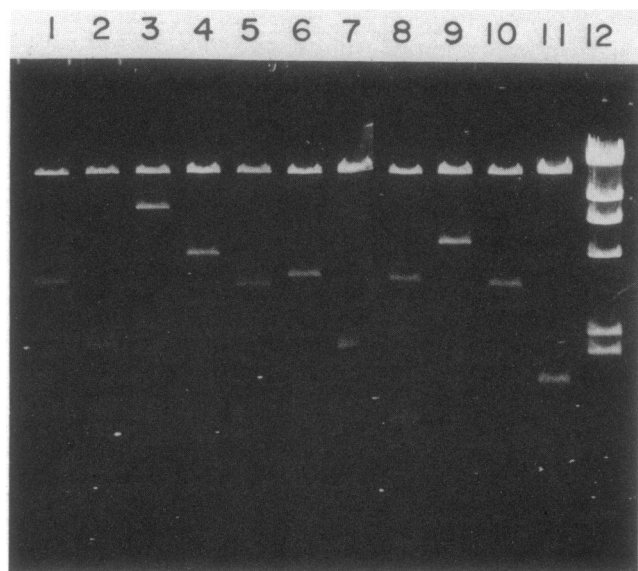


FIG. 7. Agarose gel electrophoresis of pARC plasmids in which chromosomal DNA fragments of *Streptomyces griseus* and *Streptomyces lividans*, obtained by partial digestion with *Sau*3AI, are inserted in the *Bam*HI site of pARC1. For determination of molecular sizes of inserted fragments, each plasmid was digested with *Xho*I and analyzed on a 1% agarose-ethidium bromide gel. Lanes 1 to 6 correspond to pARC9 to pARC14 containing *Streptomyces griseus* chromosomal fragments, and lanes 7 to 10 correspond to pARC15 to pARC18 containing *Streptomyces lividans* chromosomal DNA fragments. Lane 11 shows the *Xho*I digest of pARC1 as reference. Lane 12 shows λ -*Hind*III digests as standard markers (23.1, 9.42, 6.56, 4.37, 2.32, 2.03, and 0.56 kb). pARC16 (lane 8) has three *Xho*I sites, showing the presence of a *Xho*I site in the cloned fragment.

TABLE 1. Yields of pigment production by transformants carrying pARC plasmids

Plasmid	DNA source	Size (kb) of inserted DNA	Yield (OD ₄₆₀) of pigment production
pIJ41-B4	<i>aph</i> (<i>Streptomyces fradiae</i>)		0.49
pARC3	<i>ermC</i> (<i>Staphylococcus aureus</i>)	0.798	1.09 ^a
pARC4	<i>afsA</i> (<i>Streptomyces bikiniensis</i>)	0.45	0.76
pARC7	<i>tac</i> (<i>E. coli</i>)	0.092	0.28
pARC9	Chromosomal DNA from <i>Streptomyces griseus</i>	1.8	0.64
pARC10		0.5	0.62
pARC11		5.5	1.44
pARC12		2.6	0.52
pARC13		1.6	2.66
pARC14		1.8	1.54
pARC15	Chromosomal DNA from <i>Streptomyces lividans</i>	0.5	1.09
pARC16		2.9	0.36
pARC17		2.5	2.07
pARC18		1.7	1.54

^a This is the value obtained from induced cells of *Streptomyces lividans* (pARC3) grown in the presence of 5 μ g of lincomycin per ml.

base sequence GATC, and used to transform protoplasts of *Streptomyces lividans* TK21. Approximately 1 of 120 thio-strepton-resistant transformants thus obtained produced the brown pigment to some extent.

More than 50 pigment-producing transformants with which pigment production could be detected after 3 days of growth at 30°C on Bennett agar medium containing thiostrepton were obtained in the experiment involving *Streptomyces griseus* chromosomal DNA, and more than 100 pigment-producing transformants were obtained in the experiment involving *Streptomyces lividans* chromosomal DNA. After 7 days of growth, in addition to the above transformants, several colonies produced a detectable amount of the pigment in both experiments. We chose six and four pigment-producing transformants obtained in the experiments with chromosomal DNA of *Streptomyces griseus* and *Streptomyces lividans*, respectively, which were judged to produce a relatively large amount of the pigment after 3 days of growth; and purified plasmid DNAs. By restriction analysis, all of the purified plasmids contained DNA fragments with various sizes ranging from 0.5 to 5.5 kb (Fig. 7).

The amounts of pigment produced by the transformants varied, probably owing to different promoter activities of the inserted DNA fragments. A relative pigment productivity of each transformant was determined as described above. Table 1 summarizes yields of the pigment production of the transformants, together with those obtained from the transformants carrying pIJ41-B4 with the *aph* promoter sequence, pARC3 with the *tac* promoter, and pARC7 with the *ermC* promoter and regulatory region, for comparison. The table also includes a yield of the pigment directed by pARC4 which contains a promoter region of *afsA* (unpublished data), an A-factor biosynthetic gene of *Streptomyces bikiniensis* (19). Among these plasmids, pARC13 with a 1.6-kb *Streptomyces griseus* chromosomal DNA fragment caused the highest productivity, ca. 5 times as high as pIJ41-B4 with the promoter of neomycin phosphotransferase gene.

DISCUSSION

Synthesis of the brown pigment could be detected in *Streptomyces lividans* only when a DNA fragment with a promoter activity was inserted in the correct orientation to the cloned gene(s) in plasmid pARC1. Plasmid pARC1 proved useful as a probe to isolate DNA fragments with promoter activities. Since three different promoters (*aph*, *tac*, and *ermC*), all of which were excised by cleavage with restriction endonucleases, were capable of promoting expression of the cloned pigment gene(s), the translational signals including a translational initiation sequence and a ribosome-binding site are probably present in the cloned fragment. This may account for the reasonable frequency at which pigment-producing transformants appeared in the shotgun cloning of *Streptomyces* chromosomal DNA fragments.

Pigment production directed by each of the plasmids described here differed from plasmid to plasmid. The *tac* promoter with the *E. coli* "consensus" sequence, which functions as a strong promoter in *E. coli*, caused a relatively low pigment production in comparison with other promoters originated from *Streptomyces* spp. The RNA polymerase binding consensus sequences of *Streptomyces* genes may have extremely high G+C content and therefore the *Streptomyces* transcriptional machinery recognizes the *E. coli* promoters only at a low efficiency. The absence of expression of the *Saccharomyces cerevisiae* *GAL7* promoter in *Streptomyces lividans* may be ascribed to a great divergency from *Streptomyces* transcriptional signals, although the *GAL7* promoter was capable of promoting expression of the β -galactosidase gene fused downstream from it in *E. coli* (T. Yamashita, unpublished data).

As promoter strengths are expected to be associated with yield of pigment production, one can monitor the activities of a promoter at different stages of growth. By the use of this promoter-probe vector, one can determine at what stage a particular promoter functions in a complex *Streptomyces* cell cycle. Some of the transformants in which pigment production was only detected after a longer incubation may contain promoters on pARC1 that are only capable of promoting transcription in the late stage of growth. An additional advantage to using this vector would be that it facilitates studies on promoter function; promoter activities of sequences manipulated by in vitro mutagenesis would be easily estimated by measuring optical density values after a defined incubation.

We could isolate transcriptional control signals from *Streptomyces* chromosomal DNA. Ten isolates described here began to produce the pigment at a very early stage of growth and apparently continued the production, which led to a large quantity of pigment production. These promoters can be used as a source for further manipulation of *Streptomyces* genes. It would be expected that when "strong" promoter signals are attached to some antibiotic synthetic genes or structural genes for some useful enzymes, yields of the antibiotics or enzymes may be enhanced. In addition, if all genes necessary for biosynthesis of a certain antibiotic would depend upon such a promoter for transcription, production of the antibiotic might begin at an early stage of growth.

The excised *ermC* sequence promoted expression of the cloned pigment gene(s). Induction specificity of pigment production by addition of the three MLS antibiotics was different from that in the original host *Staphylococcus aureus* and in *B. subtilis*. According to the postulated induction

model (13, 21, 22), erythromycin causes erythromycin-sensitive ribosomes to stall at specific locations in the control peptide coding sequence, leading to translation of the adenine methylase mRNA by erythromycin-resistant ribosomes. The system requires nonmethylated ribosomes that undergo a conformational change when erythromycin binds, which results in stalling at the control peptide mRNA. Based on this model, it is relevant to point out that the same MLS resistance determinant can express two different phenotypes in two different host backgrounds. Hardy and Haefeli (15) reported that clindamycin instead of erythromycin had a more effective inducing activity in *E. coli*. The difference in induction specificity of *ermC* in *Streptomyces lividans* from that in the original *Staphylococcus aureus* host might be explained, if one assumes that there are differences in ribosome structure between *Staphylococcus* spp. and *Streptomyces* spp.

An additional point to be noted is that *Streptomyces lividans* TK21 is resistant to MLS antibiotics at a low level. By the antibiotic disk assay (10), no combination of two of the three MLS antibiotics used in this paper showed a distorted inhibition zone indicative of induction (data not shown). It is not clear whether the resistance is mediated by an adenine methylase or whether the addition of some MLS antibiotic induces synthesis of the methylase leading to an increased population of MLS-resistant ribosomes in this host organism. The *ermC* promoter including the control region was apparently induced by the addition of optimum concentrations of erythromycin or lincomycin, irrespective of the host backgrounds. The optimum concentration of antibiotics for induction is supposedly determined by both the population of MLS-sensitive ribosome capable of stalling at the control leader sequence of *ermC* mRNA and the concentration of MLS antibiotics that inhibits protein synthesis of the host cell.

Preliminary characterization of the brown pigment suggested that it was a shunt product of the actinorhodin biosynthetic pathway. We cloned *Streptomyces coelicolor* A3(2) chromosomal DNA fragments greater than 30 kb in length covering the pigment gene(s) by using a λ cosmid cloning system in *E. coli* (unpublished data). Restriction analysis suggested that the region surrounding the pigment gene(s) described here is similar to that of actinorhodin biosynthetic genes reported by Malpartida and Hopwood (28). Neither *Streptomyces coelicolor* A3(2) nor *Streptomyces lividans* produced a detectable amount of the brown pigment by preliminary analysis with thin-layer chromatography. In *Streptomyces coelicolor* A3(2), the brown pigment might be converted to another product too rapidly to be detected. In *Streptomyces lividans*, which was shown to contain a sequence considerably homologous to the cloned pigment gene(s), the corresponding gene(s) might not be expressed in some way. Further characterization of the pigment gene(s) is now in progress, together with determination of the structure of the pigment.

Since the brown pigment is a "secondary metabolite," it is conceivable that its biosynthesis may be affected by physiological conditions including composition of medium and flux of intracellular metabolites. In light of that, the promoter-probe vector pARC1 with the pigment production gene(s) is probably different from other probes with an antibiotic resistance gene (2) and with the β -galactosidase gene (5). On the other hand, the pigment is presumably biosynthesized via a polyketide which we assume is present in almost all *Streptomyces* spp. Therefore, it is likely that this promoter-probe gene(s) can be used in various *Strep-*

tomyces spp. to isolate promoters and characterize them by standardizing the cultures.

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