CLONING OF DNA FRAGMENTS CONTAINING *STREPTOMYCES* PROMOTER ACTIVITY

TAICHI MANOME and EMIKO HOSHINO

Fermentation Research Laboratories, Sankyo Co., Ltd.,
1-2-58 Hiromachi, Shinagawa-ku, Tokyo 141, Japan

(Received for publication February 4, 1987)

The thiostrepton-resistance gene is expressed in *Streptomyces jumonjinensis* [16]-8 SANK 61185 carrying the plasmid pIJ702 but not the tyrosinase gene. We have isolated DNA fragments from various streptomycetes that restore expression of the tyrosinase gene on pIJ702 in this organism. The nucleotide sequences of two of these DNA fragments show that they contain putative ribosome binding sites and −10 regions of possible promoters.

Development of host-vector systems for streptomycetes has enabled the cloning of genes for antibiotic-resistance[1~5], antibiotic-biosynthesis[6~10] and various enzymes[11~15]. Interest in the transcriptional regulatory regions of the cloned genes is heightened by the high G+C content of the chromosomal DNA of this group of microorganisms. Promoter-probe vectors have been constructed to clone DNA fragments containing promoter activity[16~19], and detailed analyses of several promoters have revealed the existence of common base sequences in their −10 regions in several cases[20].

Streptomyces jumonjinensis [16]-8 SANK 61185, a producer of the β-lactam antibiotics cephamycin C and clavulanic acid, was transformed with the plasmid pIJ702[12], and the transformants found to express thiostrepton-resistance (*tsr*) but not melanin productivity. *Streptomyces lividans* TK21, transformed with the pIJ702 isolated from a *S. jumonjinensis* [16]-8 SANK 61185 transformant, expressed the *tsr* gene and the tyrosinase (*mel*) gene. Therefore, the Mel⁻ phenotype of *S. jumonjinensis* transformants were not due to deletion of the *mel* gene or loss of pIJ702. It is also known that the *mel* gene on pIJ702 is not expressed in *Streptomyces clavuligerus*[12]. Clarification of the reason why the *mel* gene is not expressed in *S. jumonjinensis* [16]-8 SANK 61185 might be useful for the elucidation of the mechanism of gene expression in streptomycetes.

We were able to clone several DNA fragments from various streptomycetes, that restored the expression of the *mel* gene on pIJ702 in *S. jumonjinensis*. This paper presents the procedure used for cloning these DNA fragments, the distribution of such DNA fragments in various streptomycetes, and the analysis of the DNA sequences of two such fragments with apparent promoter activity.

Materials and Methods

Bacterial Strains and Plasmid

The plasmid-free *S. jumonjinensis* [16]-8 SANK 61185 derived from *S. jumonjinensis* SANK 66782 and *S. lividans* TK21[21] were used as the hosts for cloning and retransformation with the recombinant plasmids. The strains used as DNA donors were *Streptomyces venezuelae* KCC 526, *Streptomyces fradiae* NRRL B-1195, *Streptomyces kanamyceticus* ATCC 21252, *Streptomyces niveus* SANK 92870, *Streptomyces erythraeus* NRRL 2338, *Streptomyces rimosus* ATCC 10970, *Streptomyces omiyaensis* NRRL B-1587, *Streptomyces griseus* SANK 62457; *S. jumonjinensis* SANK 66782, SANK 67882, SANK 67182 and [16]-8 SANK 61185; *Streptomyces flavovirens* SANK 61784 and *Streptomyces* sp. SANK 61884. The plasmid pIJ702[12] was prepared from *S. lividans* 3131 kindly supplied by Dr. E.

KATZ.

Media, Growth Condition and DNA Preparation

Streptomyces strains for DNA preparation and *S. jumonjinensis* [16]-8 SANK 61185 for protoplasting were grown in GGC^{y} medium composed of glycerol 0.4%, glycine 0.1%, yeast extract (Difco) 0.1%, Casamino Acids 0.4%, $MgSO_4 \cdot 7H_2O$ 0.1%, $CaCl_2 \cdot 2H_2O$ 0.01% and trace salts solution[22] 4%. Two ingredients, KH_2PO_4 0.2% and $Na_2HPO_4 \cdot 12H_2O$ 0.8% were separately sterilized and added to this medium. GGC^{y} medium containing sucrose 34% was used to grow cells for the protoplasting of *S. lividans* TK21. Protoplast regeneration was performed in R2MP medium containing yeast extract (Difco) 0.2%, malt extract (Difco) 0.5%, L-tyrosine 0.1% and DL-norleucine 0.05% in R2 medium[23].

To grow mycelia of *Streptomyces* strains, a 5-ml seed culture in GGC^{y} medium was inoculated into 100 ml of fresh GGC^{y} medium in a 500-ml Sakaguchi flask, which was cultivated on a reciprocal shaker at 28°C for 1 day.

Total DNA was prepared as described by MARMUR[24] and plasmid DNA was isolated as described OKANISHI *et al.*[25].

Protoplast Transformation

The plasmid pIJ702 (1 μg) and donor DNA (5 μg) were mixed and digested with a restriction enzyme (*Sph* I, *Bgl* II or *Sac* I) in 50 μl of digestion buffer at 37°C for 2 hours according to the supplier's instructions. Ligation was performed with T4 DNA ligase at 14°C for 16 hours.

The optimized procedure for transformation of *S. jumonjinensis* was as follows: The mycelial pellet was harvested by centrifugation at 3,500 rpm for 10 minutes followed by washing 3 times with P medium[23] and resuspension in 20 ml of P medium. One-ml of a 40 mg/ml lysozyme solution was added to the cell suspension and the mixture was incubated at 28°C for 60 minutes with occasional shaking until completion of protoplast formation as judged by microscopic observation. After transferring the mixture into a 50-ml centrifuge tube 25330 (Corning) in ice, 2 ml of 1 M $CaCl_2$ was added and the mixture was filtered twice through a glass filter. The filtrate was centrifuged at 3,500 rpm for 10 minutes at 4°C and the protoplasts were washed once by resuspension in 20 ml of cooled Pca80 medium (sucrose 12%, NaCl 0.41%, $MgCl_2 \cdot 6H_2O$ 0.21%, $CaCl_2 \cdot 2H_2O$ 1.18% (80 mM) and Tris 0.57% (25 mM, pH 7.2)). This washing procedure was repeated and the protoplasts were resuspended in 3 ml of the same medium. For transformation the pellet obtained after centrifugation of this protoplast suspension (1 ml) was resuspended in 50 μl of 2× concentrated Pca80 medium and then the plasmid DNA in 50 μl distilled water and 500 μl of 20% polyethylene glycol (PEG) 1540 in Pca80 medium were added. After addition of the PEG solution, transformation was terminated by the addition of 5 ml of Pca80 medium. Protoplasts were collected by centrifugation and resuspended in 1 ml of Pca80 medium. A portion (0.1 ml) of the protoplast suspension was spread on R2MP regeneration plates. After incubation at 28°C for 20~22 hours, the regeneration plates were overlaid with 3 ml of R2 soft agar medium (0.5% agar) containing enough thiopeptin (purified from Thiofeed and used instead of thiostrepton after confirmation of their cross resistance) to give a final concentration of 25 μg/ml in the plate. The thiopeptin-resistant (Thio^{r}) transformants appeared after incubation at 28°C for about 1 week. Transformation of *S. lividans* TK21 protoplasts was carried out as described by THOMPSON *et al.*[26].

Nucleotide Sequence Analysis

The recombinant plasmid was completely digested with *Sph* I and the insert containing the promoter region was recovered by agarose gel electrophoresis and gel electroelution. The fragment was subcloned into M13mp18 by screening white colonies of *Escherichia coli* JM105 transformants for ones with the correct insert. Single-strand DNA was isolated and sequenced by the Sanger dideoxy method[27] using a 15-base sequence primer (Pharmacia) and [α-^{32}P]dCTP (Amersham). The fragment was sequenced in both directions. The reaction mixture were separated on 7.5% polyacrylamide sequencing gels followed by exposure to X-ray film for autoradiography.

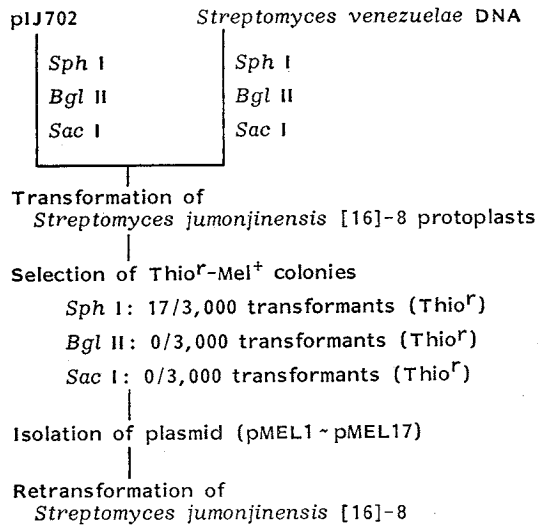
Results

Cloning and Analysis of Recombinant Plasmids Obtained from Transformants

Chromosomal DNA from *S. venezuelae* KCC 526 was used in the first experiment. When the DNA fragments digested with *Sph* I were inserted into the *Sph* I site of pIJ702, melanin production (Mel^+) was noted in 17 out of approximately 3,000 $Thio^r$ colonies. No $Thio^r$-Mel^+ transformants were obtained by using *Bgl* II or *Sac* I as the digesting enzyme (Fig. 1). To confirm the melanin production, the 17 transformants were grown on ISP 6 and ISP 7 agar plates and a strain-to-strain variation in melanin productivity was observed (data not shown). *Sph* I-digestion of the recombinant plasmids isolated from these transformants showed that they contained insert DNA fragments with various lengths from 240 to 4,100 base pairs (bp). The structure of one of these plasmids (pMEL16) is shown in Fig. 1. Similar results were obtained with clones from 10 other species (13 strains) of streptomycetes. These clones contained plasmids with 130 to 9,000 bp inserts and $Thio^r$-Mel^+ transformants were obtained at a frequency of 0.4~2.3% (Table 1).

Fig. 1. Cloning procedure and schematic diagram of pMEL16 plasmid.

The thickened line indicates the position of DNA fragment inserted into pIJ702.



S. jumonjinensis [16]-8 SANK 61185 was retransformed with these recombinant plasmids. All the transformants obtained were $Thio^r$-Mel^+ whereas the pIJ702 transformant was only $Thio^r$-Mel^- (Fig. 2).

Relation between Orientation of DNA Fragments and Expression of the *mel* Gene

When present in pIJ702 *Sph* I DNA fragments of 130 or 240 bp resulted in the transformation of *S. jumonjinensis* [16]-8 SANK 61185 to Mel^+ inspite of the fact that the fragments were too short to code the structural gene for melanin production. This result suggested that these small DNA fragments had promoter activity, which had caused expression of the *mel* gene in pIJ702 in the *S. jumonjinensis* transformants. If true, the plasmids with an opposite orientation of the inserts should lose their ability to express the *mel* gene.

The plasmids pSLF16-1, pSLF2-20 and pSLF22-11 possessing the inversely inserted DNA fragments relative to the mel^+ plasmids, pMEL16 (with a 240 bp insert), pMEL2 (1,650 bp insert),

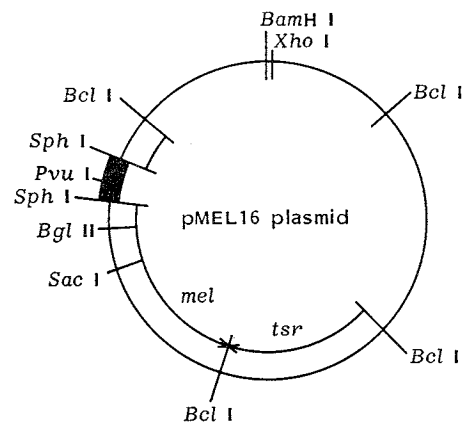
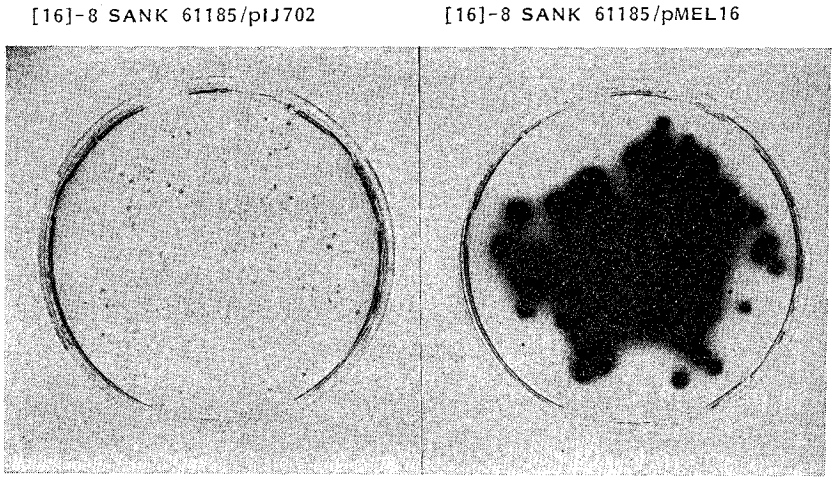


Table 1. Distribution of DNA fragments enabling *mel* gene expression.

Donor strain of DNA	Mel^+ frequency ($Thio^r$-Mel^+/ $Thio^r$-Mel^-)	Plasmid	Size of DNA inserted (bp)
Streptomyces venezuelae KCC 526	17/3,000	pMEL1～17	240～4,100
Streptomyces sp. SANK 61884	7/300	pMEL18～24	130～1,540
S. flavovirens SANK 61784	3/450	pMEL25～27	130～?
S. jumonjinensis SANK 66782	5/440		
S. jumonjinensis SANK 67882	3/278		
S. jumonjinensis SANK 67182	2/183	pMEL101～119	230～9,000
S. jumonjinensis [16]-8 SANK 61185	9/882		
S. fradiae NRRL B-1195	3/576	pMEL201～203	260～560
S. kanamyceticus ATCC 21252	1/145	pMEL301	230
S. niveus SANK 92870	1/207	pMEL401	200
S. erythraeus NRRL 2338	1/254	pMEL501	620
S. rimosus ATCC 10970	2/236	pMEL601～602	490～600
S. omiyaensis NRRL B-1587	2/241	pMEL701～702	590～?
S. griseus SANK 62457	1/135	pMEL801	1,600
11 species 14 strains	0.4～2.3%		130～9,000

Fig. 2. Melanin productivity of *Streptomyces jumonjinensis* [16]-8 SANK 61185 transformed with pIJ702 and pMEL16.

The colonies were grown on R2MP regeneration plates.



and pMEL22 (950 bp insert), respectively, were prepared. *S. jumonjinensis* [16]-8 SANK 61185 and *S. lividans* TK21 were transformed with these plasmids, and the relationship between orientation of the DNA insert and expression of the *mel* gene was studied (Fig. 3). *S. jumonjinensis* [16]-8 SANK 61185 was not Mel^+ when transformed with pIJ702, pSLF16-1, pSLF2-20 and pSLF22-11, but was when transformed pMEL16, pMEL2 and pMEL22. *S. lividans* TK21 was Mel^+ when transformed with pIJ702, pMEL16, pMEL2 and pMEL22, but not with pSLF16-1, pSLF2-20 and pSLF22-11. It is therefore obvious that expression of the *mel* gene on pIJ702 depended on the orientation of the inserted DNA fragments in *S. jumonjinensis* [16]-8 SANK 61185 and in *S. lividans* TK21.

Fig. 3. Effect of orientation of the inserted DNA on expression of the *mel* gene.

Open boxes indicate the insert DNA, thickened solid lines indicate the *mel* gene, and thin lines indicate pIJ702 DNA. The fragment sizes are given in base pairs.

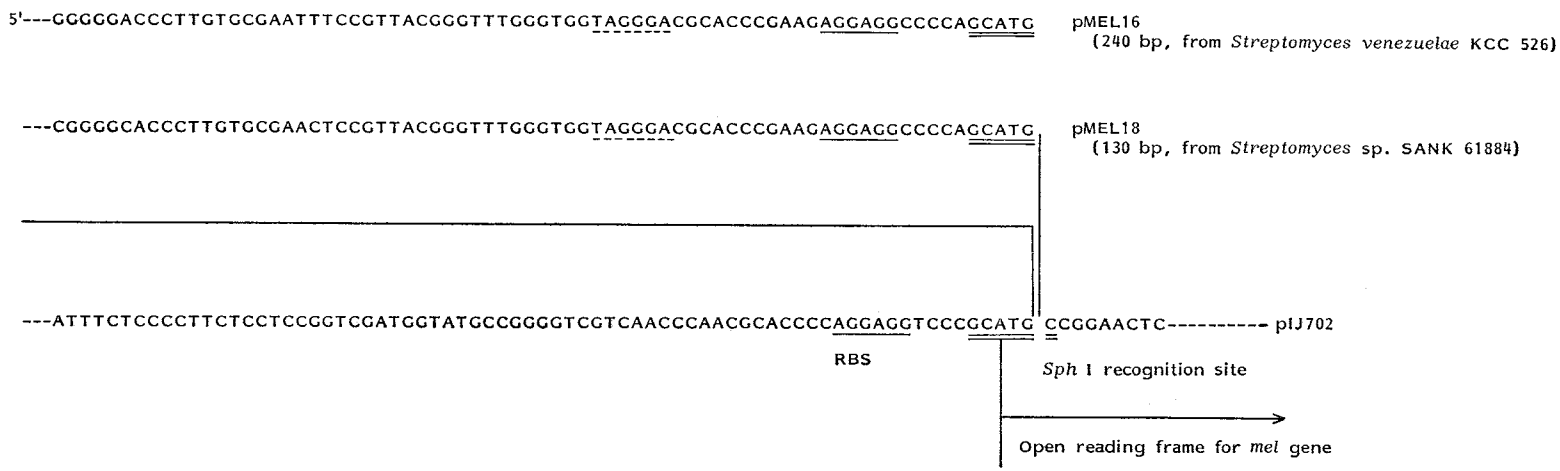
Orientation of the inserted DNA of pMEL16 and pSLF16-1 was determined from sizes of fragments generated on *Pvu* I digestion (A). Orientation of the inserted DNA of pMEL2 and pSLF2-20, and pMEL22 and pSLF22-11, respectively, was determined from sizes of fragments generated on *Sac* I or *Bgl* II digestions (B) and (C).

Plasmid	Expression of *mel* gene in *Streptomyces jumonjinensis* [16]-8 SANK 61185	Expression of *mel* gene in *Streptomyces lividans* TK21
pIJ702	−	+
pMEL16	+	+
pSLF16-1	−	−
pMEL2	+	+
pSLF2-20	−	−
pMEL22	+	+
pSLF22-11	−	−

Base Sequence of the Inserted DNA Fragments

The inserted DNA fragments of pMEL16 (240 bp insert) and pMEL18 (130 bp insert), selected for their strong inducing activity for melanin production and the short length of their DNA inserts, were sequenced and the sequence data compared with the DNA sequence near the *Sph* I site of the *mel* gene on pIJ702[28] (Fig. 4). The ATG of the *Sph* I recognition site was found to correspond to translational initiation codon of the *mel* gene. These three DNA fragments contain a AGGAGG sequence that is characteristic for a ribosome binding site (RBS) at a position 7~8 bases upstream

Fig. 4. Alignment of promoter region.
Dotted lines indicate the position of the similar sequence in −10 region of *tsr* P1 promoter.



from ATG of the *Sph* I recognition site, GCATGC. However, the DNA sequence of the *mel* gene and those of pMEL16 and pMEL18 are different in the region upstream from the putative RBS. There exists an identical sequence, TAGGGA, in the latter two plasmids that is similar to the sequence in −10 region of P1 promoter of the *tsr* gene[20] but this type of sequence is not present in the *mel* gene[28].

Discussion

DNA fragments complementary for expression of the *mel* gene in *S. jumonjinensis* [16]-8 SANK 61185 were cloned from the total DNA of various streptomycetes. Analyses of these DNA fragments showed that (1) Mel^+ strains resulted only when the *Sph* I site of pIJ702 was used as the cloning site, (2) under these conditions, DNA fragments capable of transforming of *S. jumonjinensis* [16]-8 SANK 61185 to Mel^+ were isolated from the total DNA of various streptomycetes at a high frequency (0.4~2.3% of the transformants), (3) fragments of 130 or 180 bp, too short to code an intact *mel* gene, were capable of transforming the host to Mel^+, (4) the orientation of the fragments was critical for expression of the *mel* gene, and (5) the melanin productivity of the clones grown on ISP medium varied with the cloned fragment.

Analysis of the DNA sequences of two of these DNA fragments (the inserts of plasmids pMEL16 and pMEL18) were carried out to compare them with the sequence in the vicinity of *Sph* I site of the *mel* gene in pIJ702[28]. The characteristic AGGAGG sequence, which is a putative RBS because it corresponds to the base sequence of the 3′-end region of 16s RNA from *Streptomyces* and *Bacillus*[16], was observed at 7~8 bases upstream of the ATG of the *Sph* I recognition site. These two DNA fragments possessed a nearly identical sequence, in spite of their difference in origin, and contained the TAGGGA sequence 12 bases upstream from the putative RBS, which is similar to the sequence in the −10 region of the *tsr* P1 promoter[20]. None of these features are found in the sequence of the *mel* gene in pIJ702[28].

It is clear from our data that insertion of DNA fragments with recognizable promoter regions into *Sph* I site of the *mel* gene on pIJ702 in the correct orientation created a translational fusion, resulting in expression of the *mel* gene. The reason why the *mel* gene is not expressed in *S. jumonjinensis* [16]-8 SANK 61185 may be due to heterogeneity of the RNA polymerase holo enzymes, as in *Bacillus* sp.[29] and *Streptomyces coelicolor*[30], if it lacks the sigma factor required for recognition of the *mel* gene promoter normally carried by pIJ702.

Acknowledgments

We thank Dr. E. KATZ, Georgetown University, U.S.A., for his kind gift of *Streptomyces lividans* 3131 harboring pIJ702. We thank Dr. M. ARAI, Director of Fermentation Research Laboratories, Sankyo Co., Ltd., for his encouragement throughout this work.

References

1) BIBB, M. J.; J. L. SCHOTTEL & S. N. COHEN: A DNA cloning system for interspecies gene transfer in antibiotic-producing *Streptomyces*. Nature 284: 526~531, 1980
2) THOMPSON, C. J.; T. KIESER, J. M. WARD & D. A. HOPWOOD: Physical analysis of antibiotic-resistance genes from *Streptomyces* and their use in vector construction. Gene 20: 51~62, 1982
3) MURAKAMI, T.; C. NOJIRI, H. TOYAMA, E. HAYASHI, K. KATSUMATA, H. ANZAI, Y. MATSUHASHI, Y. YAMADA & K. NAGAOKA: Cloning of antibiotic-resistance genes in Streptomyces. J. Antibiotics 36: 1305~1311, 1983
4) NAKANO, M.; H. MASHIKO & H. OGAWARA: Cloning of the kanamycin resistance gene from a kanamycin-producing *Streptomyces* species. J. Bacteriol. 157: 79~83, 1984
5) KOBAYASHI, T.; T. UOZUMI & T. BEPPU: Cloning and characterization of the streptothricin-resistance gene which encodes streptothricin acetyltransferase from *Streptomyces lavendulae*. J. Antibiotics 39: 688~693, 1986
6) FEITELSON, J. S. & D. A. HOPWOOD: Cloning of a *Streptomyces* gene for an O-methyltransferase involved in antibiotic biosynthesis. Mol. Gen. Genet. 190: 394~398, 1983

7) MALPARTIDA, F. & D. A. HOPWOOD: Molecular cloning of the whole biosynthetic pathway of a *Streptomyces* antibiotic and its expression in heterologous host. Nature 309: 462~464, 1984
8) OHNUKI, T.; T. IMANAKA & S. AIBA: Self-cloning in *Streptomyces griseus* of an *str* gene cluster for streptomycin biosynthesis and streptomycin resistance. J. Bacteriol. 164: 85~94, 1985
9) SANZAK, R.; P. MATSUSHIMA, R. H. BALZ & R. N. RAO: Cloning and expression in *Streptomyces lividans* of clustered erythromycin biosynthesis genes from *Streptomyces erythraeus*. Bio/Technology 4: 229~232, 1986
10) MURAKAMI, T.; H. ANZAI, S. IMAI, A. SATOH, K. NAGAOKA & C. J. THOMPSON: The bialaphos biosynthetic genes of *Streptomyces hygroscopicus*: Molecular cloning and characterization of the gene cluster. Mol. Gen. Genet. 205: 42~50, 1986
11) ROBBINS, P. W.; D. F. WIRTH & C. HERING: Expression of the *Streptomyces* enzyme endoglycosidase H in *Escherichia coli*. J. Biol. Chem. 256: 10640~10644, 1981
12) KATZ, E.; C. J. THOMPSON & D. A. HOPWOOD: Cloning and expression of the tyrosinase gene from *Streptomyces antibioticus* in *Streptomyces lividans*. J. Gen. Microbiol. 129: 2703~2714, 1983
13) IKEDA, H.; E. T. SENO, C. J. BRUTON & K. F. CHATER: Genetic mapping, cloning and physiological aspects of the glucose kinase gene of *Streptomyces coelicolor*. Mol. Gen. Genet. 196: 501~507, 1984
14) DEHOTTEY, P.; J. DUSART, C. DUEZ, M. V. LENZINI, J. A. MARTIAL, J. M. FRERE, J. M. GHUYSEN & T. KIESER: Cloning and amplified expression in *Streptomyces lividans* of a gene encoding extracellular β-lactamase from *Streptomyces albus* G. Gene 42: 31~36, 1986
15) MCKILLOP, C.; P. ELVIN & J. KENTEN: Cloning and expression of an extracellular α-amylase gene from *Streptomyces hygroscopicus* in *Streptomyces lividans* 66. FEMS Microbiol. Lett. 36: 3~7, 1986
16) BIBB, M. J. & S. N. CHOEN: Gene expression in *Streptomyces*: Construction and application of promoter-probe plasmid vectors in *Streptomyces lividans*. Mol. Gen. Genet. 187: 265~277, 1982
17) HORINOUCHI, S. & T. BEPPU: Construction and application of a promoter-probe plasmid that allows chromogenic identification in *Streptomyces lividans*. J. Bacteriol. 162: 406~412, 1985
18) WARD, J. M.; G. R. JANSSEN, T. KIESER, M. J. BIBB, M. J. BUTTNER & M. J. BIBB: Construction and characterization of a series of multi-copy promoter-probe plasmid vectors for *Streptomyces* using the aminoglycoside phosphotransferase gene from Tn5 as indicator. Mol. Gen. Genet. 203: 468~478, 1986
19) BRAWNER, M. E.; J. I. AUERBACH, J. A. FORNWALD, M. ROSENBERG & D. P. TAYLOR: Characterization of *Streptomyces* promoter sequences using the *Escherichia coli* galactokinase gene. Gene 40: 191~201, 1986
20) JANSSEN, G. R.; M. J. BIBB, C. P. SMITH, J. M. WARD, T. KIESER & M. J. BIBB: Isolation and analysis of Streptomyces promoters. *In* Microbiology — 1985. *Ed.*, L. LEIVE, pp. 392~396, American Society for Microbiology, Washington, D.C., 1985
21) HOPWOOD, D. A.; T. KIESER, H. M. WRIGHT & M. J. BIBB: Plasmids, recombination and chromosome mapping in *Streptomyces lividans* 66. J. Gen. Microbiol. 129: 2257~2269, 1983
22) OKANISHI, M. & K. F. GREGORY: Methods for the determination of deoxyribonucleic acid homologies in *Streptomyces*. J. Bacteriol. 104: 1086~1094, 1970
23) OKANISHI, M.; K. SUZUKI & H. UMEZAWA: Formation and reversion of streptomycete protoplasts: Cultural condition and morphological study. J. Gen. Microbiol. 80: 389~400, 1974
24) MARMUR, J.: A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. 3: 208~218, 1961
25) OKANISHI, M.; T. MANOME & H. UMEZAWA: Isolation and characterization of plasmid DNAs in actinomycetes. J. Antibiotics 33: 88~91, 1980
26) THOMPSON, C. J.; J. M. WARD & D. A. HOPWOOD: Cloning of antibiotic resistance and nutritional genes in streptomycetes. J. Bacteriol. 151: 668~677, 1982
27) SANGER, F.; S. NICKLEN & A. R. COULSON: DNA sequencing with chainterminating inhibitors. Proc. Natl. Acad. Sci. U.S.A. 74: 5463~5467, 1977
28) BERNAN, V.; D. FILPULA, W. HERBER, M. BIBB & E. KATZ: The nucleotide sequence of the tyrosinase gene from *Streptomyces antibioticus* and characterization of the gene product. Gene 37: 101~110, 1985
29) DOI, L.: Multiple RNA polymerase holoenzymes exert transcriptional specificity in *Bacillus subtilis*. Arch. Biochem. Biophys. 214: 772~782, 1982
30) WESTPHELING, J.; M. RANES & R. LOSICK: RNA polymerase heterogeneity in *Streptomyces coelicolor*. Nature 313: 22~27, 1985