

## Cloning and Characterization of a Heterologous Gene Stimulating Antibiotic Production in *Streptomyces lividans* TK-24

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Genetic determinant for the secondary metabolism was studied in heterologous expression in *Streptomyces lividans* TK-24 using *Streptomyces griseus* ATCC 10137 as a donor strain. Chromosomal DNA of *S. griseus* was ligated into the high-copy number *Streptomyces* shuttle plasmid, pWHM3, and introduced into *S. lividans* TK-24. A plasmid clone with 4.3-kb *Bam*HI DNA of *S. griseus* (pMJJ201) was isolated by detecting for stimulatory effect on actinorhodin production by visual inspection. The 4.3-kb *Bam*HI DNA was cloned into pWHM3 under the control of the strong constitutive *ermEp*\* promoter in both directions (pMJJ202; *ermEp*\* promoter-mediated transcription for coding sequence reading right to left; pMJJ203; *ermEp*\* promoter-mediated transcription for coding sequence reading left to right) and reintroduced into *S. lividans* TK-24. The production of actinorhodin was markedly stimulated due to introduction of pMJJ202 on regeneration agar. The introduction of pMJJ202 also stimulated production of actinorhodin and undecylprodigiosin in submerged culture employing the actinorhodin production medium. Introduction of pMJJ203 resulted in a marked decrease of production of the two pigments. Nucleotide sequence analysis of the 4.3-kb region revealed three coding sequences: two coding sequences reading left to right, ORF1 and ORF2, one coding sequence reading right to left, ORF3. Therefore, it was suggested that the ORF3 product was responsible for the stimulation of antibiotic production. The C-terminal region of ORF3 product showed a local alignment with Myb-related transcriptional factors, which implicated that the ORF3 product might be a novel DNA-binding protein related to the regulation of secondary metabolism in *Streptomyces*.

**Key words:** *Streptomyces griseus*, heterologous expression, *ermEp*\* promoter, stimulation of antibiotic production.

*Streptomyces* spp. are well known for their capacity to synthesize an enormous variety of antibiotics as secondary metabolites. For most cases, a strain of *Streptomyces* has capacity to produce structurally unrelated secondary metabolites, and more than 10 biosynthetic steps are required to convert primary metabolites into the final product, which imply that this organism contains complex genetic determinations. Studies on the biosynthesis of each antibiotic have revealed involvement of pathway-specific regulatory genes, the most of which are found adjacent to the biosynthetic structural gene clusters and serve as activator of the biosynthetic structural genes (18, 26). In *Streptomyces coelicolor* A3(2), *actII-ORF4* product acts as a pathway-specific acti-

vator for actinorhodin (10), as *redD* and *redZ* do for undecylprodigiosin (28, 33).

The individual biosynthetic pathway of antibiotics has been implicated under the pleiotropic regulation. Several pleiotropic regulatory loci were revealed in *S. coelicolor* A3(2) that produces four structurally unrelated antibiotics including actinorhodin and undecylprodigiosin (2, 5, 8, 9, 22). The known pleiotropic regulators include signal-transducing proteins which employ protein phosphorylation for their activities (27): AfsK-AfsR, eukaryotic type serine-threonine kinase (13, 19); AfsQ1-AfsQ2, two-component regulatory proteins (16); PtpA, phosphotyrosine protein phosphatase (30). Other than these kinase- and phosphatase-encoding regulatory genes, diverse genetic determinants were implicated in the regulation of antibiotic production. The diversity of these regulatory elements makes it likely that several mechanisms

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might be involved in regulating antibiotic production. The combination of all these regulatory mechanisms, and the still unknown connections between different levels of regulations in *Streptomyces*, make this topic challenging for further understanding of the control of secondary metabolites production.

In efforts to understand the complex network of the regulation in antibiotic production of *Streptomyces*, new DNA sequences were examined for ability to stimulate actinorhodin production in *Streptomyces lividans* whose ability to produce actinorhodin was normally "silent". This attempt resulted in isolation of several new regulatory genetic loci; *abaA* (9), *abaB* (25), a gene encoding a putative antisense regulator (22). In the present study, we reported isolation of *Streptomyces griseus* DNA which stimulated actinorhodin production in *S. lividans* TK-24. Phenotypic studies implicated that there were isolated the genes for positive and negative regulation of antibiotic production in the region of 4.3-kb *Bam*HI DNA from *S. griseus* ATCC 10137.

## Materials and Methods

### Bacterial strains and plasmids

*Escherichia coli* DH5 $\alpha$  (23), pUC18 (32), and pBluescript KS(+) (Stratagene, La Jolla, Calif.) plasmids were used for routine subcloning. *S. lividans* TK-24 (14) and *E. coli* DH5 $\alpha$  were used as hosts for the high-copy number *Streptomyces* shuttle vector pWHM3 (31) or for their derivative plasmids (Table 1).

### DNA isolation, manipulation, and cloning.

*S. griseus* ATCC 10137 was the original source of genomic DNA for the cloning experiments. Procedures for manipulation of *Streptomyces* and general recombinant DNA manipulation were as described elsewhere (14, 23). Protoplasts of *S. lividans* TK-24 was transformed using the procedures of Hunter (15). For the *Streptomyces* vector selection, thiostrepton was used at 50  $\mu$ g/ml in agar and

10 g/ml in broth cultures.

### Assay of actinorhodin and undecylprodigiosin

Actinorhodin production medium (17) contained (per liter) glycerol, 50 g; glutamic acid, 5 g; morpholinopropane sulfonic acid, 21 g; MgSO<sub>4</sub>·7H<sub>2</sub>O, 200 mg; CaCl<sub>2</sub>·2H<sub>2</sub>O, 100 mg; NaCl, 100 mg; KH<sub>2</sub>PO<sub>4</sub>, 82 mg; FeSO<sub>4</sub>·7H<sub>2</sub>O, 9 mg and trace element solution (14), 2 mL at a final pH 6.5. Fifty milliliters of the media were contained in a 250-mL baffled flask and incubated at 28°C with a shaking speed at 250 rpm. The media were inoculated with spores and mycelium from plate cultures of the recombinant strains of *S. lividans* TK-24 on R2YE agar (14). To prepare vegetative inocula, the cells from R2YE agar were added to 50 mL of R2YE medium in 250-mL baffled flask. The cultures were incubated for 72 h at 28°C at a shaking speed at 250 rpm; the mycelium obtained by centrifugation was washed, resuspended in the original volume of water, and was used to inoculate the production medium. Actinorhodin content and growth were determined following the method described by Liao *et al.* (17) and undecylprodigiosin content by Narva and Feitelson (20).

### DNA sequencing and analysis

The nucleotide sequence was determined in both directions by the dideoxynucleotide chain termination method (24), using double-stranded plasmid DNA and the universal primers. DNASIS software (Hitachi) was used for sequence analysis. The codon usage pattern was determined by FRAME analysis (3). The Fasta3 program at the European Bioinformatics Institute (21) and the Blast program at the National Center for Biotechnology Information (1) were used to search for local alignment.

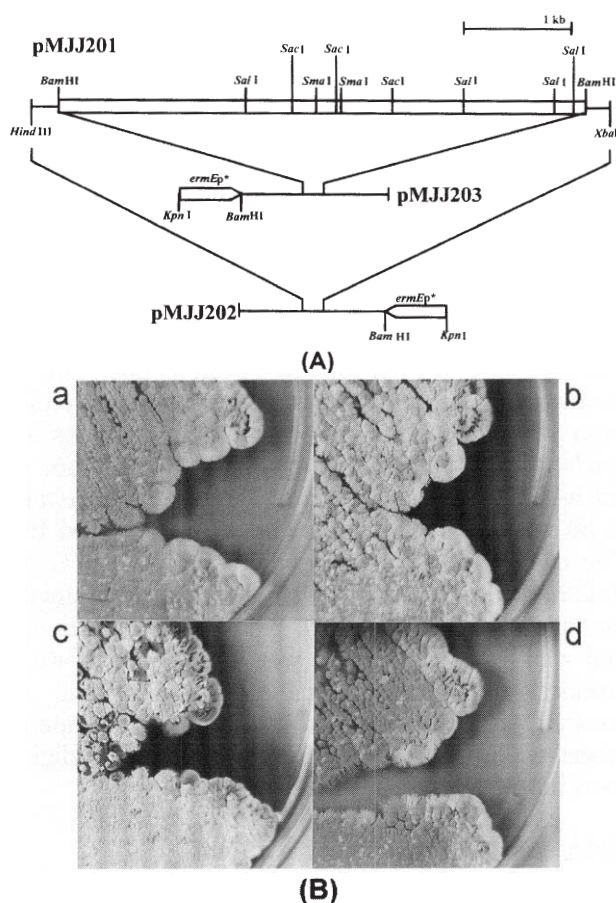
## Results

### Cloning and characterization of the DNA that stimulated actinorhodin production in *S. lividans* TK-24

Table 1. Plasmids used in this study

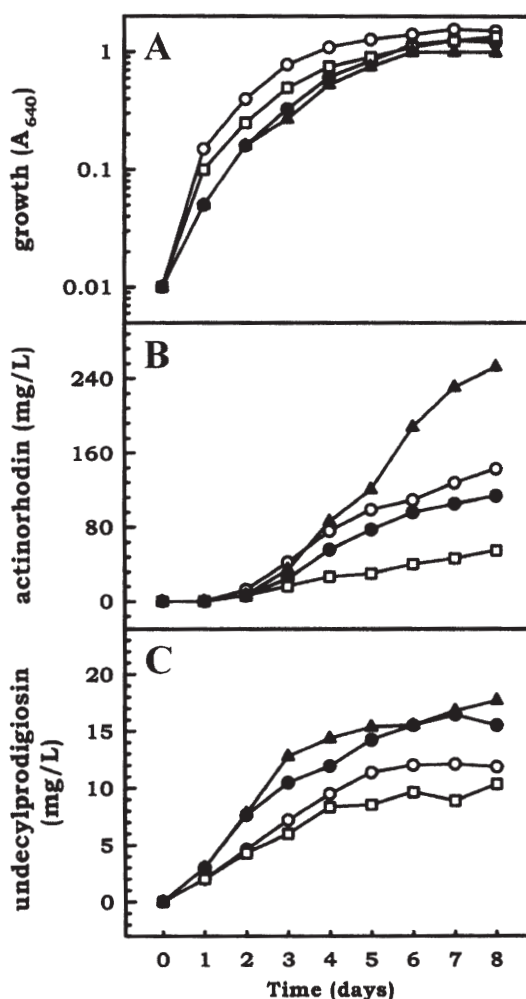
Plasmid	Genotype	Reference or Sources
pIJ4026	A derivative of pUC18 with a 1.7-kb DNA fragment; <i>ermE</i>	4, 29
pMJJ201	A derivative of pWHM3 containing 4.3-kb <i>Bam</i> HI DNA from <i>S. griseus</i>	This work
pMJJ200	A derivative of pWHM3 containing 279-bp <i>Kpn</i> I- <i>Bam</i> HI fragment from pIJ4026	This work
pMJJ102	pBluescript KS(+) containing 4.3-kb <i>Xba</i> I- <i>Hind</i> III fragment from pMJJ201	This work
pMJJ103	pBluescript KS(+) containing 4.3-kb <i>Bam</i> HI fragment from pMJJ201 at the opposite direction of pMJJ102	This work
pMJJ202	A derivative of pMJJ200 containing 4.3-kb <i>Xba</i> I- <i>Hind</i> III fragment from pMJJ102	This work
pMJJ203	A derivative of pMJJ200 containing 4.3-kb <i>Xba</i> I- <i>Hind</i> III fragment from pMJJ103	This Work





**Fig. 1.** (A) Restriction map of the 4.3-kb *Bam*HI region. The significant restriction endonuclease sites are noted below. The plasmids derived from pMJJ201 (Table 1) were shown. (B) Activation of actinorhodin production due to introduction of pMJJ201 or pMJJ202 on R2YE agar. The recombinant *S. lividans* TK-24 strains grown on R2YE agar at 28°C for 7 days. **a**, **b**, **c**, and **d** indicate *S. lividans* TK-24 harboring pMJJ200, pMJJ201, pMJJ202, and pMJJ203, respectively.

The DNA fragment between approximately 4.0-kb and 6.0-kb in *Bam*HI-digested chromosomal DNA of *S. griseus* ATCC 10137 was recovered from the gel, purified, and ligated into the high-copy number *Streptomyces* shuttle vector pWHM3. The ligation mixture was introduced by transformation into *S. lividans* TK-24, with selection of thiostrepton resistance. Among the transformants, an intensively blue colony was isolated. Analysis of plasmid DNA (named as pMJJ201) from this colony revealed 4.3-kb insert in the cloning site of the vector (Fig. 1A). The cloned DNA was subcloned into pMJJ200, a derivative of pWHM3 containing *ermEp\** promoter, in both directions (named as pMJJ202 and pMJJ203, respectively) and reintroduced into *S. lividans* TK-24 (Fig. 1A). As shown in Fig. 1B, *S. lividans* TK-24 transformed with pMJJ201 or pMJJ202 showed blue pigment production, whereas the strain



**Fig. 2.** Growth (A) and production of actinorhodin (B) and undecylprodigiosin (C) of *S. lividans* TK-24 transformed with pMJJ200-203 in the actinorhodin production medium. ○, ●, ▲, and □ indicate strains harboring pMJJ200, pMJJ201, pMJJ202, and pMJJ203, respectively. The cultures were prepared in the production medium with inocula of spores and mycelium from plate cultures of R2YE agar. The cells were cultured in 50 mL of the broth in a 250 ml-baffled flask at 28°C with a shaking speed at 250 rpm.

harboring pMJJ200 or pMJJ203 showed negligible pigment production. The strain with pMJJ202 showed more intense color development than the strain with pMJJ201. On visual inspection, the strains with pWHM3 and pMJJ200 showed no difference in the pigment production on R2YE agar.

*S. lividans* TK-24 transformed with the pWHM3 derivatives were cultured in R2YE broth, and productions of actinorhodin and undecylprodigiosin were examined. In the R2YE broth culture, *S. lividans* TK-24 could not produce actinorhodin. However, actinorhodin was produced up to 18.5 mg/L in *S. lividans* TK-24 harboring pMJJ202. The growth of the strain harboring pMJJ202 was significantly

retarded compared to other recombinant strains consistent with the growth-interference of pMJJ202-introduction detected by visual inspection on R2YE agar. In the culture condition employed, the growth of the strain with pMJJ202 measured as 10 mg/mL in dried cell weight, whereas those of other recombinant strains measured 18 to 22 mg/mL.

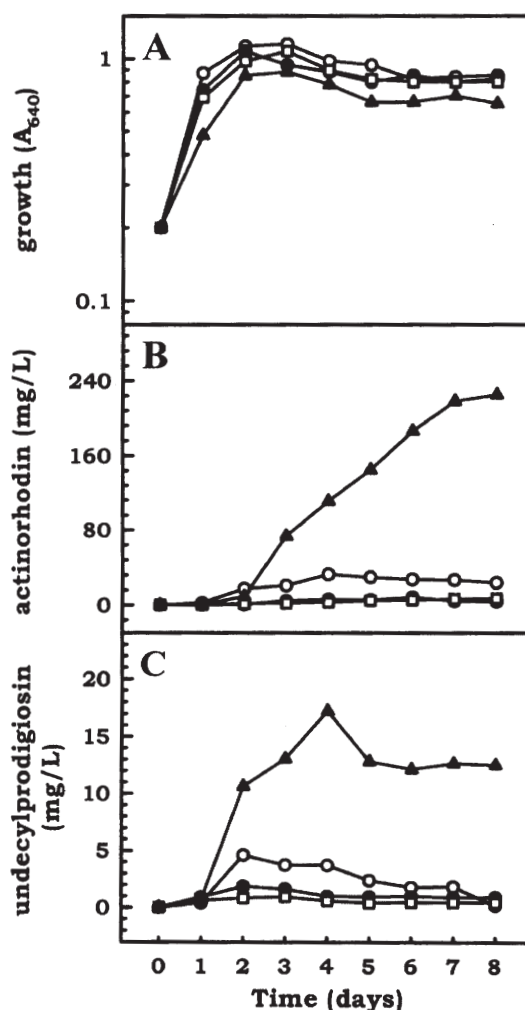
### Phenotypic studies of the recombinant *S. lividans* TK-24

The productions of actinorhodin and undecylprodigiosin were examined in actinorhodin production medium permitting the substantial accumulation of both pigmented antibiotics. The cell growth decreased considerably by introduction of the 4.3-kb DNA (Fig. 2A). Compared to *S. lividans* TK-24 harboring pMJJ200, actinorhodin production increased up to 180% in the strain harboring pMJJ202 at 8 days after initiation of the cultures, and the enhancement was clearly observed at 4 to 6 days (Fig. 2B). Actinorhodin production was somewhat low in the strain harboring pMJJ201 and markedly repressed in the strain harboring pMJJ203 compared to the strain harboring pMJJ200; actinorhodin content in the strain harboring pMJJ203 measured only a third of that detected in the strain harboring pMJJ200 at 8 days after initiation of the cultures. Undecylprodigiosin production of the strains with pMJJ202 and pMJJ201 increased upto 150% and 130% of that of the strain harboring pMJJ200, respectively (Fig. 2C). The introduction of pMJJ203 lowered undecylprodigiosin content, but the decrement was relatively low compared to that observed in actinorhodin production (Fig. 2B and C).

When productions of actinorhodin and undecylprodigiosin were examined in the cultures inoculated with mycelium from R2YE broth culture, marked differences in the productivities were observed (Fig. 3B and C). Whereas the production of the pigmented antibiotics of the strain with pMJJ200 was significantly limited, compared to the productivity observed in Fig. 2, the pigments were markedly overproduced due to introduction of pMJJ202 to an extent obtainable in expt of Fig. 2. The pigment production of the strain harboring pMJJ201 or pMJJ203 measured as a basal level (Fig. 3B and C).

### Nucleotide Sequence Analysis

Computer-aided FRAME analysis (3) with the nucleotide sequence of the 4.3-kb *Bam*HI DNA predicted putative three complete coding sequences (Fig. 4). The average G+C content for third codon position for each coding sequences were 87.6, 82.1, and 92.0%, respectively. There were two reasonable



**Fig. 3.** Growth (A) and production of actinorhodin (B) and undecylprodigiosin (C) of *S. lividans* TK-24 transformed with pMJJ200-203 in the actinorhodin production medium. ○, ●, ▲, and □ indicate the strains harboring pMJJ200, pMJJ201, pMJJ202, and pMJJ203, respectively. The cells were cultured in 50 ml of R2YE broth in a 250 ml-baffled flask for 3 days at 28°C with a shaking speed at 250 rpm and used to inoculate the production medium to be approximately 0.1 of  $A_{640}$ . The inoculated production mediums were maintained at the same culturing condition.

candidates for the translational start codon of ORF1, ATG (nt 261 to 263) and TTG (nt 279 to 281). By finding Shine-Dalgarno sequence located at nt 273 to 276 (GAGG), a TTG codon at nt 279 to 281 was assigned as the translation start site. ORF1 was predicted to terminate at a TGA codon at nt 1710 to 1712. Comparison of the ORF1 product with known proteins revealed that the ORF1 product showed regional similarity to Hsp70 proteins, a family of proteins highly conserved in evolution (6, 7); The ORF1 product was most similar to Hsp70 protein of *S. griseus* 2247 (11), with an



**BamHI**

GGATCCCCGT	GACCGGCCTG	CGCCTCGCGA	CGGTGCTGAG	CGGATCGGCG	CGCATTGGGA	60
CCCGGCGGAC	GAGTTCCGCG	GCGGCTCTCC	CTGGCCGGAG	CGCTCACCAC	TCTCATCCAG	120
CGGCACGCAC	GCCCCCGACC	GCCCCCTIACA	CCGAAGCCGC	CGGGGCCACG	GCGCCGCCGG	180
ACGCGCGACC	GAACGCGGCG	CGGCGGGCGC	GTCCGGCCGT	CGGCATCGAC	TTCGGGACGA	240
<b>rbs</b>						
CGAACTCGGC	GGTCGCCGTC	ATGGAGGGCG	CGGAGGTCTT	GTTGATCCCC	AACGCCCAGG	300
GCA3GCATAC	CACTCCCAGC	CTGGTGGCCC	TCACCGCCGA	GGGGGACGCG	CTGGTCGGCA	360
R H T T P S	L V A L	T A E	G D A	L V G T		27
CGGACGCCGA	CGGGCAGGCC	CTTGCCAACC	CCGGCTTCAC	CGCGGGCGCC	GCCATGCTGT	420
D A E R Q A	L A N P	G F T	A G A	A M L W		47
GGCTGGGCAC	CGACTGGCGT	GTGCGCGGGG	GCGGGGTACG	GCTGACCGCC	GAGGACGTCG	480
L G T D W R	V A R G	G V R	L T A	E D V A		67
CCG3GCTGGT	CCTCGCCGSC	CTGCGCGAGG	ACGCCGAGGC	CTACCTCGGC	GAGCCGGTCA	540
G L V L A R	L R E D	A E A	V L G	E P V T		87
CCGACGCGGT	GCTGGCGGTG	CCGCTGGGTT	TCCGTGCGGA	CCAGCGTGCC	GCGCTGGGGG	600
D A V L A V	P A G F	R R D	Q R A	A L G A		107
CCCGCGGTGA	ACGGGCGGGG	CTCAACGTTT	TGCGGCTGGT	CAACGTGCCT	ACGGCCGTGG	660
R G E R A G	L N V L	R L V	N V P	T A V A		127
CGACGTCTTA	CGGGCCGAAC	CGGGACGACC	TCAGTGTCTT	GGTCTTCGAT	CTCGGCCGGG	720
T S Y G P N	R D D L	T V L	V F D	L G G G		147
GCACCTCTGA	CGTCTCCCTC	ATCGAACTCG	GTGACGGCGT	CGTGGAGATC	AGGGCCACGC	780
T L D V S L	I E L G	D G V	V E I	R A T P		167
CGGCGACAGC	CGGCTCGGSC	GGCAACGACT	GGGACCGAGC	GATCCTCGAG	CACCTGACCG	840
A D S R L G	G N D W	D Q R	I V E	H L T D		187
ACCATGTGCG	CGGGCGGCAC	GGGCTGGATC	TCACCGCCGA	TGTCGCCGCG	ATCCAGCGGC	900
H V R R R H	G V D L	T G D	V A A	I Q R L		207
TGCGCGAGGC	GGCCGAGAGC	GCAAGGATCG	AACTGTGCGC	GGCGCGTACC	ACGACCGTCC	960
R E A A E T	A R I E	L S A	A R T	T T V R		227
GGTCCCTTA	TCTGCCACCC	GGTCCGGACA	GCCCCGTCCA	CCTGAAGAG	GAAGTGAACC	1020
L P V L A T	G P D S	P V H	L E E	E L T R		247
GCGAGGAGTT	GGAGAGGGTC	ACCCAGGACC	TGCTGGAACG	CTGCCGAACC	CCGGTCCGAA	1080
E E L E R L	T Q D L	L E R	C R T	P V E N		267
ACGTCTCTGC	CGACGCGGGG	TGCACGCTCG	CCGATATCGA	TCAGGTCGTC	CTGACGGGCG	1140
V L A D A G	C T L A	D I D	Q V V	L T G G		287
GTGCGCCCTT	GATGCTTGGC	GTGGGCGACC	TGGTCCCGCG	GCTCACGGGC	GGCCAGGGGT	1200
A A L M P A	V G D L	V R R	L T G	G Q G S		307
CCTACGAGCG	TCTGAGGCCG	GAGGCCGTTG	TCCATGGCGC	GGTGTCTCAG	GCAGGCAATC	1260
V Q R L S P	E A V V	H G A	V L Q	A G I L		327
TGACCGGGGA	GGTGAAGGAC	GTGCTGCTCC	TGGACGTGCG	CCCTACTCG	ATCGGCGTCC	1320
T G E V K D	V L L L	D V A	P V S	A A A C		347
AAACGCACGA	CGGGACCATG	AAGAAGCTGC	TCCAGCGGAA	CACGACCATC	CCCACGAGGC	1380
I G V E T H	D G T M	K K L	L Q R	N T T I		367
GCTCGACGCT	CTTCAACACG	CACACGGACG	ACCAGCCCAT	GGTGTATTTC	CACATCGTGG	1440
P T R R S D	V F T T	H T D	D Q P	M V L F		387
AGGGCGAGCG	AAAGGACGCC	GCGCGGAACT	GGCCCCCTCG	CGTCTCTGAA	CTCGCCCTGC	1500
H I V E G E	R K D A	A R N	W P L	A V L E		407
<b>Sall</b>						
CTCCCGCTCC	TCCGGGTGIG	CCCATGATCG	AGGTGACGGT	CGACTGCACC	GCCAGCGACG	1560
L A L P P A	P R G V	P M I	E V T	V D C T		427
ATCTTCATAT	CAAGGTACAG	GATCTGGGCA	CGGGCAACGA	GACGTCCGCC	ACGGTCCGGC	1620
A S D D L H	I K V R	D L G	T S N	E T S A		447
A3GCGACGAA	GGAAAGAGCG	GCGGCGCTCC	TGCGCTCCTC	CCGCTGGGCC	CGCCTGCGTG	1680
T V G Q A T	K E R A	A A L	L R S	S R W A		467
ATCTCGTCCG	TGTACTAC	CCGGCCIGIT	GAATGCTCGT	GTTCCCGCGT	CGTACTCCAC	1740
R L R D L V	P V T H	P A C	*			480
CTCGACATCG	ACGCGATCGA	CGGAACCGGG	GGCTGCGGCG	TCGTGCCACG	GAAGGCACGT	1800
CTTCGGCACC	GGTTGCCCGG	CGGTGAGGCG	TGTCGGGCTG	TCCGCGCGCC	GCCGGTTGCG	1860
<b>SacI</b>						
TGGGGGCGCG	CCAGGAATCG	TCCAGAGTTC	GTCCAGAGGA	CGAGCTCCGG	CGCAGGGGTT	1920
<b>rbs</b>						
GTACAGTGCG	TCCTTGCGGT	TGTGCGGGGA	CGGTCTGCTG	GTGG3CGGTS	TGTTCTGGT	1980
CTATGGCGCG	TTGGAGGTGC	CAGAGGGTGA	CCGCGGGGAG	GTGTCTCCG	AGCCGGGCGG	2040
Y G A L E V P	E G D	R G E	V S S E	P G G		27
<b>SnaI</b>						
C3GCCCCGAG	GTTTTTCGACC	GCACCGGCCA	GGAGAGTCTT	GGCGGGGTGC	CCGGGGGCGC	2100
G P E V F D R	T G Q	E S P	G G V P	G G A		47
CGGGCCGGGC	G3GTGACCG	GGGTGCGCGG	GCGGGGCGGG	AACGTCCGCT	GC3GCGGTTT	2160
G P G G V T G	V A G	R G G	N V R C	G G F		67
CGAGGAGGTC	GGCGAGTTCC	GTGATGCCGG	GGTCTCGGG	TACGTGTCCG	CCCCAGCGGG	2220
E E V G E F G	D A G	V L G	V V S A	P A G		87
TGGCTGCGGT	CGGCGAGCTG	GGTGAGACGG	CGGTGCGGCG	CGGCTGCCAG	GC3TTTGGAG	2280
G L R R R A G	*					94
<b>SacI</b>						
AGCTCCCGGT	TGATCACCCC	GGGATTGTGC	CCCCAGGTCA	CGGGCGTGGT	GCCACAACCG	2340
<b>SnaI</b>						
CCCCTGCTCC	GGCGGCTGTT	GGGTCCGGTC	ACGCCGGGGC	GGTGGTCATC	GTCCGCGTCC	2400
		AG	TGCGGCCCGG	CCACCAAGTAG	CAGGCGCAGG	
			* A P A	T T M	T R T	390
TTGGCGGCGG	TGCGGGGGCG	GTGGTTCGGC	GGTGTGCGGG	GGCGGCGGGT	TCGGTGGGGG	2460
AACCGCCGCC	ACGCCCCCGC	CAACAGGCGG	CCACACGCCC	CCGCGGCCCA	AGCCACCCCC	
R P P P A P A	T T R	R H A P	A A P	E T P		370

CGGGTCCGGC	GCCCGCGGGC	TGCTTCGGTG	TGGCGGTATC	CGGGACGGTG	GTGGAGCGGG	2520
GCCAGGGCCG	CCGCGCGCCG	ACGAAGCCAC	ACCGCCATAG	GCCCTGCCAC	CACCTCGCCC	
A P G A	A A P	Q K P	T A T D	P V T	T S R	350
ACGGGGCTGC	GGTGGTGGAG	ACCGGGGGGC	CGGTGGGAG	GGGTGGGTGC	AGTGGTGTGG	2580
TGCCCCGACG	CCACCACCTC	TGCGCCCCCG	GCCACCGTC	CCCAACCAGC	TCACCACACC	
S P A A	T T S	V R P	G T P L	P Q D	L P T	330
TGAGGGCGTG	GTGGGCGGCC	AGGAGGAGGC	GGGTGGTGT	GCCGGAGAGC	GGGCGGGTGC	2640
ACTCCCGCAC	CACCGCGCGG	TCCTCCTCCG	CCCACCACTA	CGGCCTCTCG	CCCGGCCACG	
T L A H	H A A	L L L	R T T I	G S L	P G T	310
GCCAGGAGGC	GGTGTGCGGT	GCGATCCGGG	CGAGATGCGC	GGCCAGGGGC	CCGTCTGTGT	2700
CGGTCTCCG	CCACAGCCCA	CGCTAGGCC	CGTCTACGCG	CCGGTCCCCG	GGCAAGACGA	
R W S A	T D P	A I R	A L H A	A L P	G N Q	290
<i>SacI</i>						
GGGTCATCGC	GTGGGCGAGG	GCGGGCCACT	GACGGGAGCT	CACCAGGAGC	GCGGCGTCCC	2760
CCCAGTACGC	CACCCGCTCC	CGCCCGGTGA	CTGCCCTCGA	GTGGTCTCTG	CGCCGCAAGG	
Q T M R	H A L	A P W	Q R S S	V L L	A A D	270
GCTGGGGGCA	GGCTTCGTTG	ACCTGGCTCA	CCAGGGTGCG	GTGGGCGGGC	GGGGTGATGC	2820
CGACCCCGTC	CCGAAGCAAC	TGGACCGAGT	GGTCCCACGC	CACCCGCGCG	CCCCACTACG	
R Q P L	A E N	V Q S	V L T R	H A A	P T I	250
CGAGCTGTTC	CAGGGGCGCG	GCGCGGTCCG	TGAGGTTCAG	GTCTGGGGGG	ATGTGAGGGC	2880
GCTCGACAAG	GTCCCGCGCC	CGCGCCAGCG	ACTCCAGCTC	CAGCACCCCG	TACAGCTCCG	
G L Q E	L A R	A R D	S L D L	D H P	I D L	230
CTTCGGGTGAG	CGGGCCCGAG	AGGTTCTCTG	CGTCGGGCGT	CACCTCCGGA	GCGAGCGGCA	2940
GAAGCCACTC	GCCCGGGGTC	TCCAAGGAGC	GCAGCCGCGA	GTGGAGGCGT	CGCTCGCCGT	
G E T L	P G W	L N R	A D A S	V E P	A L P	210
CGGCGCGGGC	CGCGGCGGGG	GCGCGCGGTG	GGAGCGGCGG	GGGGCGGGGC	GGGCGCACGG	3000
GCGGGCGCGC	GCGCCGCGCC	CCGGCGCCAC	CCTCGCGGGC	CCCCGCCCCG	CCCGCGTGCC	
V A A A	R A P	P R P	P L P R	P R P	P R V	190
GCGCGGCGCG	GGCTGCGGGC	GCGGGCGCGG	TGGTGGGGGT	GATGGCGGTG	GCGATGGTCT	3060
CGCSCCGGCG	CCGACGCGCG	CGCCCGCGCC	ACCACCCCGA	CTACCGCCAC	CGCTACCAGA	
P A A P	A A P	A P A	T T P T	I A T	A I T	170
GCTCGATGCG	GAGGCTTGCT	GTGTGGGCGT	CGGTGAGGAT	GCGGGTGAAG	TCGATGCCCT	3120
CCAGCTACGC	CTCCGGACGA	CACACCGCGA	GCCACTCCTA	CGCCCACTGC	AGCTACGGGA	
Q D I R	L G A	T H A	D T L I	R T V	D I G	150
GGTCTGTGAG	GCGGCCCATG	GTGGCGGCGA	TGTCGGGCCA	GGTAGGGGAG	GCGAGGATCG	3180
CCAGCAOCTC	CGCCGGGTAC	CACCGCCGCT	ACAGCCCGGT	CCATCCCTTC	CGCTCCTAGC	
Q D H L	R G M	T A A	I D P W	T P S	A L I	130
CGTTCGGGAG	CAGGGCGTGC	GGGAGGGTGG	TGCGCAGGAG	ATCGGCCAC	CGGTCTGGTG	3240
GCAAGGCTGC	GTCCGGCAGC	CCCTCCCAAC	ACGCTCCTC	TAGCCGGGTG	GCCAGCCACG	
A N R V	L G D	P L T	T R L L	D A W	R D T	110
CCTCGGTCTT	CGTGCGGGCG	GTGTTGCGGG	CGACGGCGCG	CTCGACGCGT	GCGGCCATGG	3300
GGAGCCAGAA	GCACGCCCGC	CACAACGCCC	GCTGCGCGCG	GAGCTGCGGA	CGCCGGTACC	
G E T K	T R A	T N R	A V A R	E V G	A A M	90
<i>Sall</i>						
TGGCGAGGTT	CGGGAGGAAC	TGGGTGAGGT	CGACGCCGCG	GCGGCTGAGG	GAGACGAGTT	3360
ACCCTGCAAA	GCCCTCCTTG	ACCCACTCCA	GCTGCGGCGG	CGCCGACTCC	CTCTGCTCAA	
T A L N	P L F	Q T L	D V G A	R S L	S V L	70
GCGGGGCGCG	CACGGGCGAG	TGGTGGGTTG	TGGTGAAGTG	GTGAGGAGG	GAGCCGGGCA	3420
CGGCGCGGCA	GTGCGCGGTC	AGCCCCCACA	ACCACCTCAC	CAGCTCCTCC	CTCGGCCCGT	
Q R A T	V P W	D P T	N T L H	D L L	S G P	50
GGTCTGTGCC	GAGTTGCTCG	GCGGACCACC	AGGACTGCGC	CATCTGTCCG	ATGTGCTCGG	3480
CCAGHACG	GTCAAGCAGC	CGCCTGGTGG	TCCTGACGCG	GTAGACAGGC	TACAGCAGCC	
L H K G	L E D	A S W	W S Q A	M Q G	I D D	30
GCAGGTCTGT	TTCCTGGCCG	TGGTGGTGT	TCTGGGAGGC	GCGGCGGACC	GCCTCGCGCA	3540
CGTCCAGCAC	AAGGACCGGC	ACCACCAAA	AGACCTCCG	CGCCGCGCTG	CGCAGGCGCT	
P L D H	E Q G	H H H	K Q S A	R R V	A D A	10
<i>rbs</i>						
TCGTGAGGAT	CAGCCGAGCG	GACATCGCGG	CGGCGTGGCG	GGCTTCGGAG	AGGGCTTCGG	3600
AGCACTCCTA	GTGCGCTGCG	CTGTAGCGCC	GCCGGAGC			1
I T L I	L R V	S M				
AGTACGGCTC	GCCCGACAGT	TGGGCGGGCG	CGGGAGTGGT	CACTGGGGAG	GCTCCTTCGT	3660
CGTGGTCAGC	GCGAGCGCCC	CGGACCCGGC	CCCGTGGCGG	GACCTGGGGC	GGGCCCCGGA	3720
CGCGGGGCGG	GCCGGGCTGC	TGGGTGCGGT	CCGGTGGGGG	GTGCGGTGCG	TACTGCGGTA	3780
ACGGGCGGGG	GTGTGGCGGG	GCCACGGGCG	GGGGCGGGTG	TCACCGGTAC	GGTGGCGGGC	3840
GGTGTGTGTG	TGCGGGCGGG	GGCGCCGCTC	AGGTGCGCGG	CTTCGCGCAG	GAGGGCGGGC	3900
GCTTGGGCGG	CGGCTGTTGC	CTGAGTGTGC	TTCTCGTTGA	GCGGCGGGGG	GCTCAACGGG	3960
GCGGGGCGCG	CGGTCAAGTC	GGCGGCTTCG	GGCAGGAGGG	GCGGCGCCTG	GGCCCGTTGA	4020
<i>Sall</i>						
GCGGCGGGCC	GCCCGTCGAC	CGTGTCTGAA	GGTGTCCGGT	CTTCGTGAAG	GTGGCGACGT	4080
CCCGCTGATC	ATGGCGAATC	GGCTGGGCGG	GTGGGCGTTA	CGACTGGGCG	TGCGGCTCGT	4140
TGGGACTGTT	ATGGCGATGC	GTGAAGGTTT	TCGGCTCTGC	GTGATGTGCG	GGACCGGTCT	4200
<i>Sall</i>						
GCTCGGCGGG	ACGGCAATAC	TGGTCGACGG	CGCCCGCGCG	CAGGGTCTGC	GGACGCGTAC	4260
<i>BamHI</i>						
TCGCTTCCTG	ATCTGCGGGT	CCTGCTACGA	CCGAGGGTGT	CCGGATCC		4308

**Fig. 4.** Nucleotide sequence of the 4,308-bp *Bam*HI fragment. The deduced amino acid sequence of the proposed translation product is given below the nucleotide sequence. The asterisks denote translation termination codons. For ORF3 reading right to left, both strands are shown. Potential ribosomal binding sites (rbs) are noted, as are significant restriction endonuclease sites. The nucleotide sequence was deposited to the GenBank database under the accession number AF147749.



identity score of 53% and a similarity of 70% at the region of amino acid residues 26 to 472. The *hsp70* gene of *S. griseus* 2247 encodes the 67 kDa protein with 618 amino acid residues (11).

ORF2 was predicted to start at a GTG codon at nt 1961 to 1963 and terminate at a TGA codon at nt 2243 to 2245. ORF3 was predicted to start at a ATG codon at nt 3565 to 3563 and terminate at a TGA codon at nt 2371 to 2369. ORF2 and ORF3 had putative Shine-Dalgarno sequences located 8 bp upstream from the initiation codons.

## Discussion

Genetic locus for regulation of secondary metabolism was looked for by its ability to stimulate actinorhodin production in *S. lividans* TK-24. As a DNA donor, we chose *S. griseus* ATCC 10137 for its ability to produce streptomycin, which is biosynthetically unrelated to the known metabolites of *S. lividans* TK-24. We employed constitutive *ermEp\** promoter for amplified expression of coding sequences in the cloned DNA. The introduction of the 4.3-kb *Bam*HI DNA on a high-copy vector activated actinorhodin production on R2YE agar, and amplified expression through *ermEp\** promoter for coding sequence(s) reading right to left (Fig. 1A) resulted in dramatic stimulation of actinorhodin production (Fig. 1B). The result indicated that there might be a sequence activating actinorhodin production in the 4.3-kb *Bam*HI DNA, and the putative coding sequence be read right to left. The activating effect was also observed in the R2YE broth culture. However, the production of undecylprodigiosin was not increased by the introduction of the 4.3-kb *Bam*HI DNA.

We employed chemically defined medium rendering a substantial accumulation of actinorhodin together with undecylprodigiosin and investigated the effects of the 4.3-kb *Bam*HI DNA on the production of these two pigments. Undecylprodigiosin accumulated during the exponential growth phase, and actinorhodin production occurred mainly in the stationary phase (Fig. 2), which implicated that the physiological controls on the productions of the two pigments were markedly disparate. As shown in Fig. 2B and C, undecylprodigiosin production of the strain harboring pMJJ201 increased to an extent comparable to that of the strain harboring pMJJ202 but actinorhodin production was somewhat decreased by the introduction of pMJJ201. This phenomenon implicated that there may be a promoter for the coding sequence reading right to left, and the promoter was only active at the expo-

nential growth phase.

The production of the pigments was strictly limited when the actinorhodin production medium was inoculated with mycelium from R2YE broth culture (Fig. 3B and C). It was likely that the growth reached stationary phase too early that physiological signal(s) triggering gene(s) relating to the secondary metabolism failed to fully generate. This limitation in the antibiotic production was overcome by the expression of the putative coding sequence reading right to left in the 4.3-kb *Bam*HI DNA. In this culture condition permitting short-exponential growth phase, the strain with pMJJ201 showed a nearly complete inhibition in the production of the two pigments as did the strain with pMJJ203. Therefore, it was suggested that the expressions of the activating sequence (reading right to left) and the inhibiting sequence (reading left to right) were clearly disparate depending on growth stages; the former expressed mainly at the exponential growth phase and the latter did at the stationary phase.

The nucleotide sequence analysis of the 4.3-kb *Bam*HI DNA revealed one coding sequence reading right to left, ORF3, and two coding sequences reading left to right, ORF1 and ORF2. Therefore, it was suggested that the antibiotic production was regulated in positive manner by ORF3 product and in negative manner by either ORF1 or ORF2 product. However, no significant similarity to protein sequences in the data bases was observed for ORF2 and ORF3, and ORF1 showed only a regional similarity to *hsp70*.

Although no significant similarity to protein sequences in the data bases was observed for ORF3, a putative regulatory sequence which acted in positive manner, some characteristics of the deduced protein were obtained through computer-aided search for regional similarities. The regions of low-complexity ('simple sequence' or 'composition biased regions') were analyzed through PredictProtein server in EMBL (34), and the regions not marked as 'simple sequence' were separately analyzed for a local alignment using the *PSI*-Blast Program. The region of residue 264 to 385 showed a significant alignment with ALL-1 protein (a mammalian zinc finger protein; GenBank Accession No. P55200) with 23% identity, 41% similarity, and 15% gap in the alignment of 122 residues. The region of residue 215 to 293 showed a significant alignment with Myb-related transcriptional factors of various origins; most similarity with DNA-binding protein MybHv5 of *Hordeum vulgare* (GenBank Accession no S35729) with 24% identity, 40% similarity, and 7% gap in the alignment of 79 residues. Although a

putative DNA-binding motif could not be assigned, the alignments implicated that the ORF3 product might be a eukaryotic type DNA-binding protein related to the regulation of secondary metabolism. ORF1 or ORF2 product was supposed to be a negative effector of secondary metabolism. However, it could not be completely ruled out that the observed effect of the 4.3-kb DNA might have originated from the region other than the predicted coding sequences.

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