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

Hyung-Jin Kwon, Seung-Soo Lee, Soon-Kwang Hong, Uhn Mee Park[1] and Joo-Won Suh*

Department of Biological Science and Institute of Bioscience & Biotechnology, Myong Ji University, Yongin 449-728;
[1]Division of Life Science, University of Suwon, Suwon 445-743, Korea

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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 *ermE*p* promoter in both directions (pMJJ202; *ermE*p* promoter-mediated transcription for coding sequence reading right to left: pMJJ203; *ermE*p* 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 undecylproidigiosin 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, *ermE*p* 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), *act*II-ORF4 product acts as a pathway-specific activator 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

* To whom correspondence should be addressed.
(Tel) 82-335-330-6190; (Fax) 82-335-335-8249
(E-mail) jwsuh@wh.myongji.ac.kr

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 DH5α (23), pUC18 (32), and pBluescript KS(+) (Stratagene, La Jolla, Calif.) plasmids were used for routine subcloning. *S. lividans* TK-24 (14) and *E. coli* DH5α 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 μg/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; $MgSO_47H_2O$, 200 mg; $CaCl_2 \cdot 2H_2O$, 100 mg; NaCl, 100 mg; KH_2PO_4, 82 mg; $FeSO_4 \cdot 7H_2O$, 9 mg and trace element solution (14), 2 mL at a final pH 6.5. Fifty mililiters of the media were contained in a 250-mL baffled flask and incubated at 28℃ 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℃ 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; *ermE*	4, 29
pMJJ201	A derivative of pWHM3 containing 4.3-kb *Bam*HI DNA from *S. griseus*	This work
pMJJ200	A derivative of pWHM3 containing 279-bp *Kpn*I-*Bam*HI fragment from pIJ4026	THis work
pMJJ102	pBluescript KS(+) containing 4.3-kb *Xba*I-*Hind*III fragment from pMJJ201	This work
pMJJ103	pBluescript KS(+) containing 4.3-kb *Bam*HI fragment from pMJJ201 at the opposite direction of pMJJ201	This work
pMJJ202	A derivative of pMJJ200 containing 4.3-kb *Xba*I-*Hind*III fragment from pMJJ102	This work
pMJJ203	A derivative of pMJJ200 containing 4.3-kb *Xba*I-*Hind*III fragment from pMJJ103	This Work

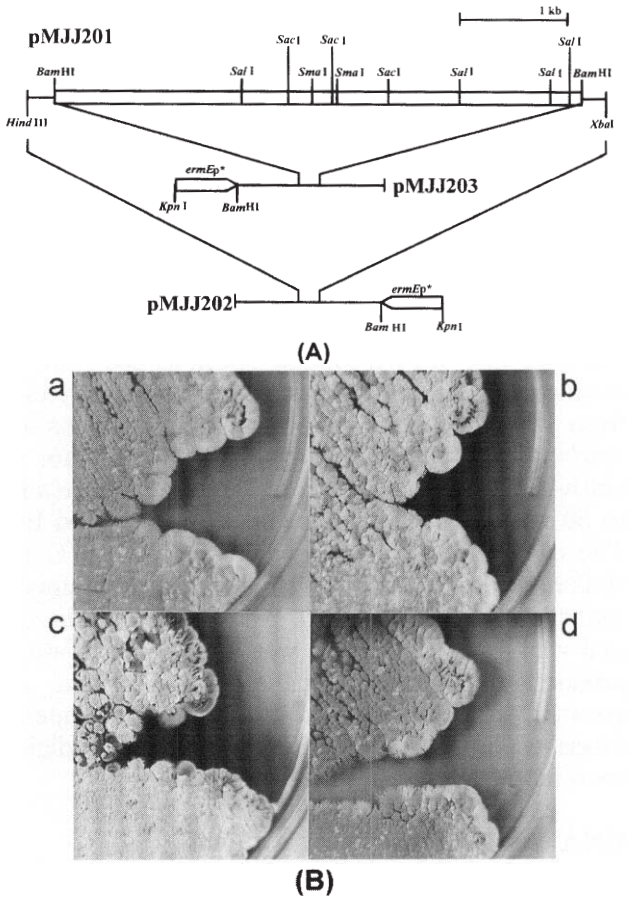


Fig. 1. (**A**) Restriction map of the 4.3-kb *Bam*HI region. The significant restriction endonuclease sites are noted below. The plasmids derived from pMJJ201 (Table 1) were shown. (**B**) Activation of actinorhodin production due to introduction of pMJJ201 or pMJJ202 on R2YE agar. The recombinant *S. 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.

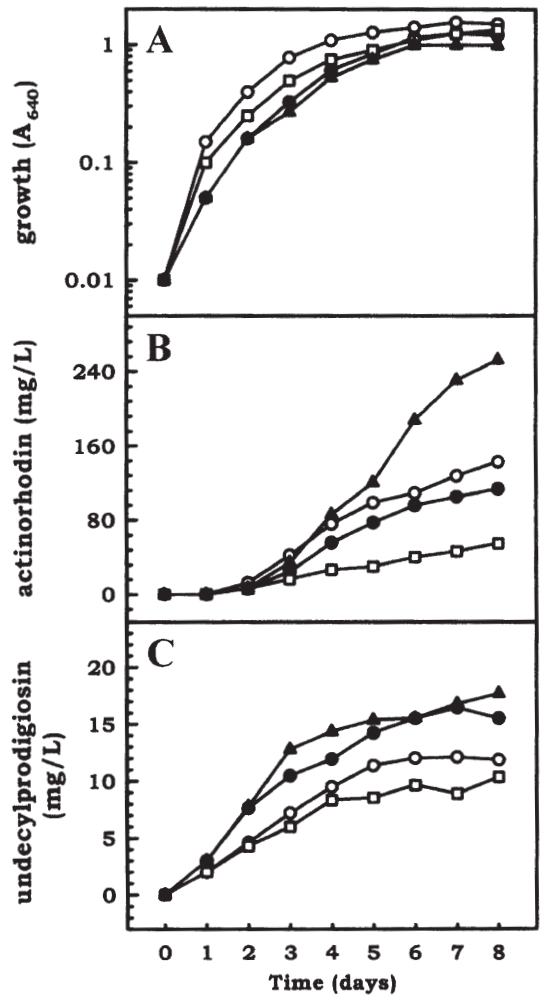


Fig. 2. Growth (**A**) and production of actinorhodin (**B**) and undecylprodigiosin (**C**) of *S. lividans* TK-24 transformed with pMJJ200-203 in the actinorhodin production medium. ○, ●, ▲, and □ indicate strains harboring pMJJ200, pMJJ201, pMJJ202, and pMJJ203, respectively. The cultures were prepared 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.

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 *ermE*p* 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 harboring pMJJ200 or pMJJ203 showed negliable 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 upto 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 pMJ203 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).

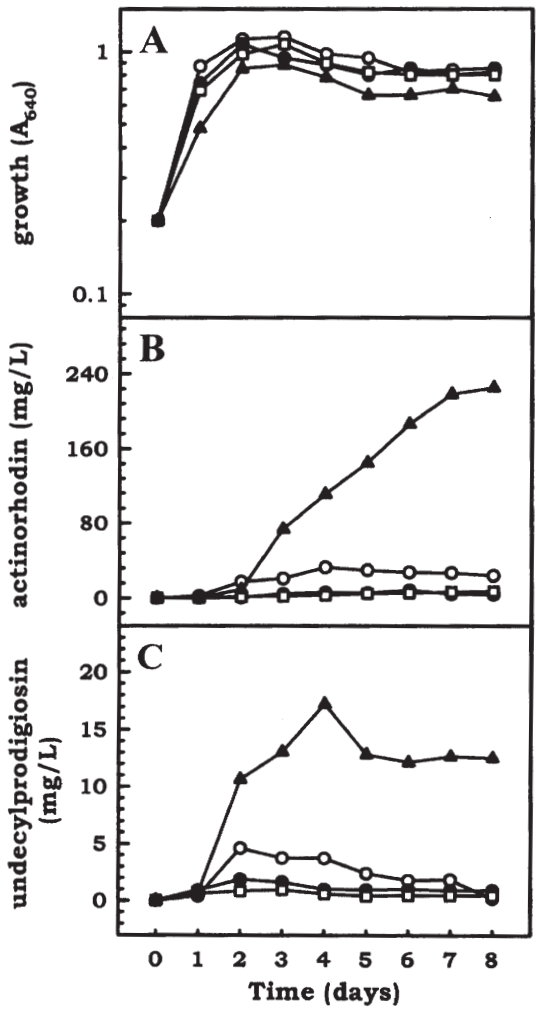
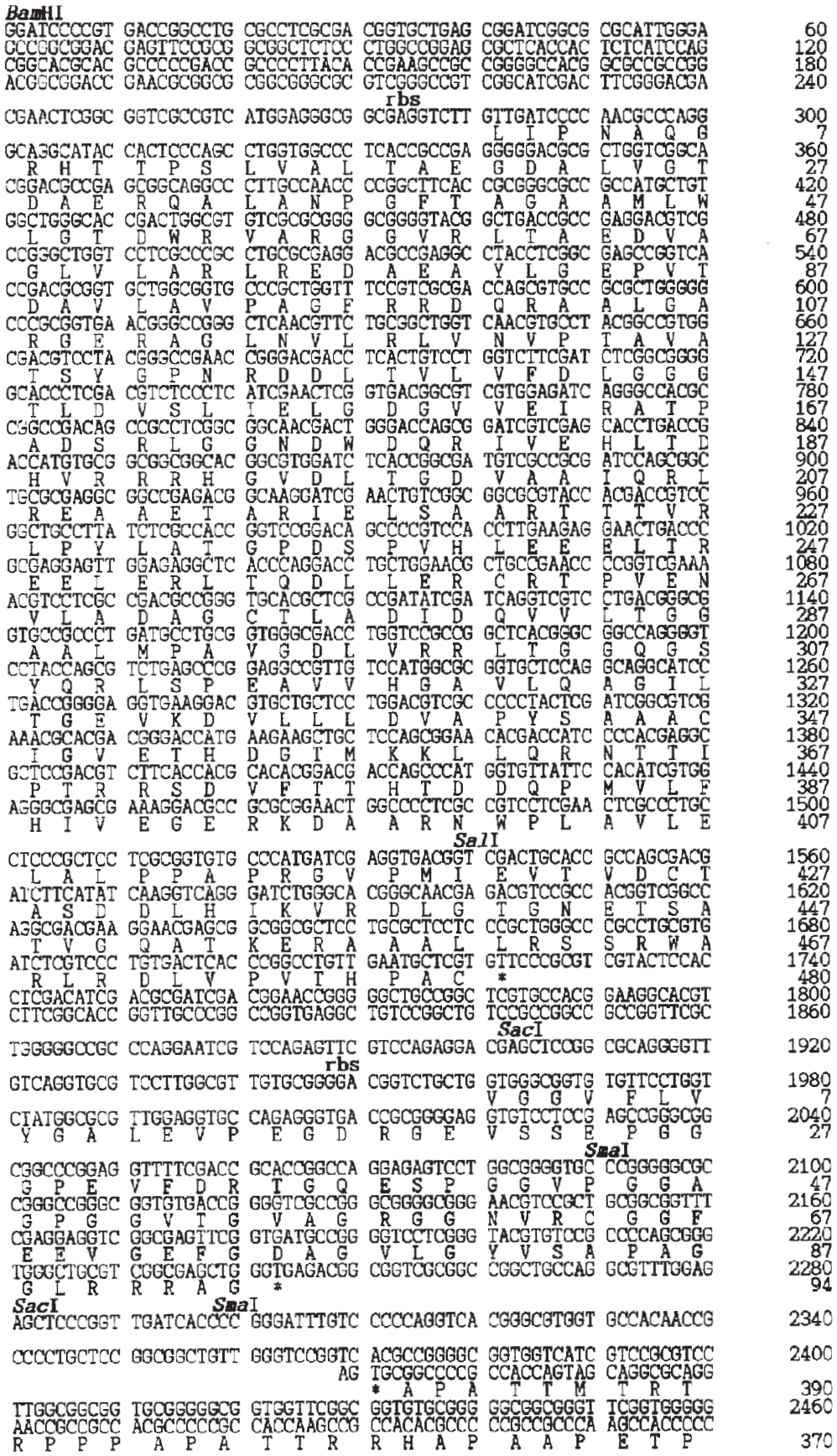


Fig. 3. Growth (A) and production of actinorhodin (B) and undecylprodigiosin (C) of *S. lividans* TK-24 transformed with pMJJ200-203 in the actinorhodin production medium. ○, ●, ▲, and □ indicate the strains harboring pMJJ200, pMJJ201, pMJJ202, and pMJJ203, respectively. The cells were cultured in 50 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.

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 candidates for the translational start codon of ORF1, ATG (nt 261 to 263) and TTG (nt 279 to 281). By finding Shine-Dalgano 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
rbs
SalI
SacI
rbs
SmaI
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SmaI

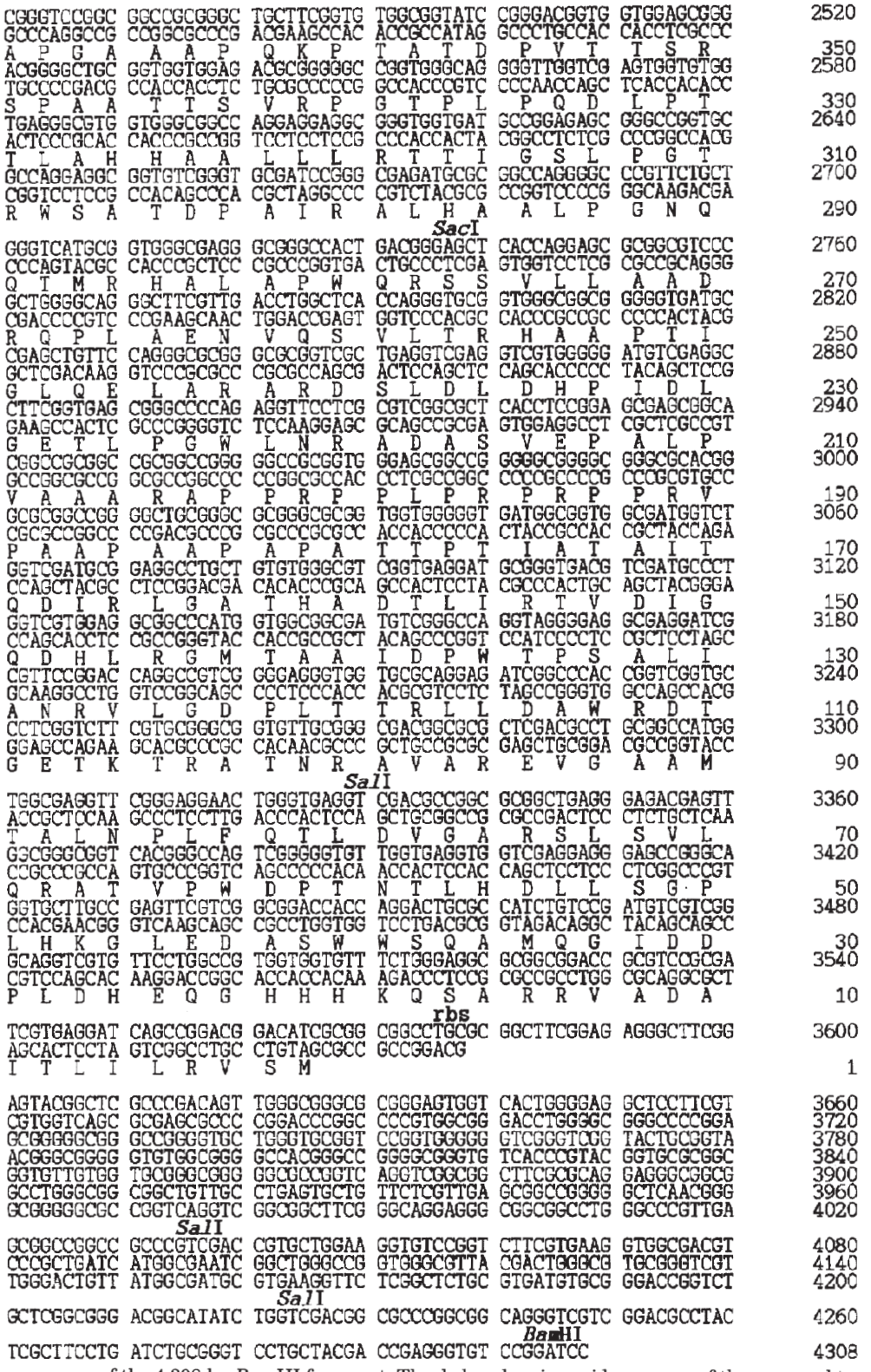

Fig. 4. Nucleotide sequence of the 4,308-bp *Bam*HI fragment. The deduced amino acid sequence of the proposed translation product is given below the nucleotide sequence. The asterisks denote translation termination codons. For ORF3 reading right to left, both strands are shown. Potential ribosomal binding 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-Dalgano 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 *ermE*p* 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 *ermE*p* 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 exponential 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.

Acknowledgments

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