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Heterologous Expression of *Streptomyces albus* Genes Linked to an Integrating Element and Activation of Antibiotic Production

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Abstract Probing *Streptomyces albus* ATCC 21838 chromosomal DNA with a proline tRNA sequence resulted in an isolation of a putative integrating element in the 6.4-kb *Eco*RI fragment. It was found that *Streptomyces lividans* TK-24 transformed with a cloned DNA fragment on a multicopy plasmid, produced a higher level of spore pigment and mycelial red pigment on a regeneration agar. Furthermore, the transformant *S. lividans* TK-24 produced a markedly increased level of undecylprodigiosin in a broth culture. A nucleotide sequence analysis of the cloned region revealed several open reading frames homologous to the integrases of integrating plasmids or temperate bacteriophages, signal-transducing regulatory proteins with a conserved ATP-binding domain, oxidoreductases (β-ketoacyl reductase), and an AraC-like transcriptional regulator. To examine the effect on antibiotic production, each coding region was overexpressed separately from the other genes in the region in *S. lividans* TK-24 with; pJHS3044 for the expression of the signal-transducing regulatory protein homologue, pJHS3045 for the homologue of oxidoreductase, and pJHS3051 for the homologue of the AraC-like transcriptional regulator. Phenotypic studies of *S. lividans* TK-24 strains harboring plasmids for the overexpression of individual genes suggested the following effects of the genes on antibiotic production: The oxidoreductase homologue stimulated the production of actinorhodin and undecylprodigiosin, which was influenced by the culture conditions; the homologue of the AraC-like transcriptional regulator was the most effective factor in antibiotic production within all the culture conditions tested; the signal-transducing regulatory protein homologue repressed the effect due to the homologue of the AraC-like transcriptional regulator, however, the antibiotic production was derepressed upon entering the stationary phase.

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Streptomyces spp. are well known for their capacity to synthesize an enormous variety of antibiotics as secondary metabolites [25, 33, 39]. In most cases, a single *Streptomyces* strain has the capacity to produce structurally unrelated secondary metabolites, and normally more than 10 biosynthetic steps are required to convert the primary metabolites into the final product, which implies a complex genetic determination [9, 21, 33]. Studies on the biosynthesis of individual antibiotics have revealed the involvement of pathway-specific regulatory genes, most of which are found adjacent to biosynthetic structural gene clusters and serve as activators of the biosynthetic pathway [36, 51]. In addition to this "local" regulation of the biosynthetic pathway, individual biosynthetic pathways are also under pleiotropic regulation [4, 14, 15]. Research efforts toward the isolation and functional characterization of the regulatory genes will increase the understanding of the mechanisms controlling antibiotic production in streptomycetes, which in turn may render a yield increase through the genetic engineering of this organism.

Streptomyces coelicolor, the principal organism for the study of streptomycete molecular genetics, produces two polyketide-derived pigments, actinorhodin and undecylprodigiosin [23]. Research into the genetic control of the antibiotic synthesis in *S. coelicolor* has attracted considerable interest since the pigment-nature of actinorhodin and undecylprodigiosin allows for a visual observation of production [48]. Spore pigmentation in *S. coelicolor* A3(2) arises from the accumulation of an unidentified grey compound. The genetic locus *whiE* specifies the pigment production, and the sequence of *whiE* indicates that the

spore pigment is biosynthesized through the polyketide pathway [12]. Results confirming the polyketide nature of the pigment were derived from the production of extracellular aromatic polyketide through the engineered expression of the *whiE* genes in *S. coelicolor* [61].

In *Streptomyces lividans*, a close relative to *S. coelicolor*, the biosynthetic pathways for actinorhodin and undecylprodigiosin are normally "silent" or expressed at very low levels. It is thus probable that a genetic determinant(s) outside the biosynthetic cluster(s) is not expressed under the normal growth conditions. Consistent with this implication, pigment production increased when a regulatory locus for antibiotic production was introduced into *S. lividans* TK-24, and the genetic determinants for antibiotic production were isolated through a heterologous expression in *S. lividans* TK-24 [15, 46, 47].

The authors have previously reported on the isolation of the polyketide biosynthetic gene cluster from salinomycin-producing *Streptomyces albus* [32] without identifying the polyketide metabolites. Accordingly, diverse approaches have been applied to clone a genetic determinant to induce the production of secondary metabolites from the gene cluster. This paper reports on the isolation of a genetic determinant for antibiotic production from *S. albus* through a heterologous expression in *S. lividans* TK-24 [30]. A nucleotide sequence analysis suggested that the DNA fragment is a part of an integrating element. A deduced amino acid sequence analysis revealed genes homologous to a putative signal-transducing regulatory protein, AraC-like transcriptional regulator, and oxidoreductase. Phenotypic studies of *S. lividans* TK-24, which harbors these genes, suggested that the cloned gene products exert a pleiotropic regulation on the production of secondary metabolites in a positive manner.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Escherichia coli DH5α [49], pUC18 [61], and pBluescript KS(+) (Stratagene, La Jolla, U.S.A.) were used for routine subcloning. *S. lividans* TK-24 [22] and *E. coli* DH5α were used as the hosts for the high-copy number *Streptomyces* shuttle vector pWHM3 [57] or for their derivative plasmids (Table 1).

DNA Isolation, Manipulation, and Cloning

S. albus ATCC 21838 was the original source of genomic DNA for the cloning experiments. Southern and colony hybridization were performed using a Hybond-N membrane (Amersham, Braunschweig, Germany) following standard procedures [49] with a digoxigenin-labeled pl-α3 probe. The DNA probe was labeled using a DIG labeling and detection kit (Boehringer-Mannheim, Mannheim, Germany). pl-α3 is a 4.5-kb DNA fragment isolated from *S. coelicolor* for its ability to restore actinorhodin and undecylprodigiosin production in *S. coelicolor abs8752* [54] and exerts a pleiotropic activation of antibiotic production when introduced into *S. lividans* TK-24 (M. Y. Choi *et al.*, 1998. *Abstr. Annu. Meet. Soc. Actinomycetes Japan*, Japan, p. 50).

Procedures for the manipulation of *Streptomyces* and general recombinant DNA manipulation have been described elsewhere [22, 49]. Protoplasts of *S. lividans* TK-24 was transformed using the procedures of Hunter [24]. For the selection of *Streptomyces* transformants, thiostrepton was used at 50 μg/ml in an agar, and 10 μg/ml in broth cultures.

DNA Sequencing and Analysis

The nucleotide sequence was determined in both directions using the dideoxynucleotide chain termination method [50], with double-stranded plasmid DNA and universal primers. DNASIS software (Hitachi) was used for the sequence analysis. The codon usage pattern was determined by a FRAME analysis [5]. The Fasta3 program at the European Bioinformatics Institute [41] and the Blast program at the National Center for Biotechnology Information [2] were used to search for a local alignment. The MultAlin program at the National Institute of Agronomic Research [11] was used for a multiple sequence alignment.

Table 1. Plasmids used in this study.

Plasmid	Genotype	Reference or source
pIJ4026	A derivative of pUC18 with a 1.7-kb DNA fragment; *ermE*	[6], [56]
pJHS11	A derivative of pUC18 containing 279-bp *Kpn*I-*Bam*HI fragment from pIJ4026	This work
pJHS14	A derivative of pWHM3 containing 0.3-kb *Eco*RI-*Bam*HI fragment from pIJ4026	This work
pJWA1	pBluescript KS(+) containing 6.4-kb *Eco*RI fragment isolated from *S. albus*	This work
pJWA2	6.4-kb *Eco*RI fragment cloned from pJWA1 into pWHM3	This work
pJHS301	1.3-kb *Sal*I-*Eco*RI fragment cloned from pJWA1 into pBluescript KS(+)	This work
pJHS305	1.3-kb *Xho*I-*Sph*I fragment cloned from pJHS301 into pJHS11	This work
pJHS3051	1.3-kb *Eco*RI-*Hind*III fragment from pJHS305 cloned into pWHM3	This work
pJHS3042	4-kb *Sph*I fragment from pJWA1 cloned into pJHS11	This work
pJHS3043	4-kb *Sph*I fragment from pJWA1 cloned into pJHS11 at the opposite direction of pHS3042	This work
pJHS3044	4-kb *Eco*RI-*Hind*III fragment from pHS3042 cloned into pWHM3	This work
pJHS3045	4-kb *Eco*RI-*Hind*III fragment from pHS3043 cloned into pWHM3	This work

Assay of Actinorhodin and Undecylprodigiosin

The actinorhodin production medium [35] contained (per l) glycerol, 50 g; glutamic acid, 5 g; morpholinopropane sulfonic acid, 21 g; $MgSO_4 \cdot 7H_2O$, 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 a trace element solution [22], 2 ml, at a final pH of 6.5. Fifty milliliters of the medium was contained in a 250-ml baffled flask and incubated at 28°C at a shaking speed of 250 rpm. The media were inoculated with spores and mycelium from a culture plate of the recombinant strains of *S. lividans* TK-24 on R2YE agar [22]. To prepare the vegetative inocula, cells from the R2YE agar were added to 50 ml of the R2YE medium in a 250-ml baffled flask. The cultures were incubated for 72 h at 28°C at a shaking speed of 250 rpm; the mycelium obtained by centrifugation was washed, resuspended in the original volume of water, and used (2%, v/v) to inoculate the production medium. The actinorhodin content and growth of the culture were determined following the method described by Liao *et al.* [35] and the undecylprodigiosin content was determined using the method of Narva and Feitelson [38].

Results and Discussion

Isolation of the DNA that Stimulated Pigment Production in *S. lividans* TK-24

A Southern blot analysis, which used the labeled pl-α3 as a probe against the *S. albus* ATCC 21838 chromosomal DNA digested with various restriction enzymes, revealed a 6.4-kb *Eco*RI fragment. This fragment was recovered from the gel, purified, ligated to pBluescript KS(+), and transformed into *E. coli* DH5α. Screening using the same probe resulted in a plasmid clone with the 6.4-kb *Eco*RI insert. To examine whether the genetic determinant for the biosynthetic pathway for secondary metabolites is located within the 6.4-kb *Eco*RI fragment, the DNA fragment was subcloned into a the high-copy number *Streptomyces* shuttle vector pWHM3 (pJWA2) and introduced into *S. lividans* TK-24 with a selection for thiostrepton resistance. The *S. lividans* TK-24 with pJWA2 showed an improved spore pigmentation and a development of the mycelial red color on the R2YE agar (Fig. 1A). To verify the ability of the 6.4-kb *Eco*RI fragment to increase the production of secondary metabolites, the undecylprodigiosin content of the recombinant strain was examined in a R2YE broth culture (Fig. 1B). *S. lividans* TK-24 and its recombinant strains cannot produce actinorhodin in a R2YE broth culture. However, as shown in Fig. 1B, the undecylprodigiosin production was enhanced up to approximately 300% due to the introduction of pJWA2, which suggested that a genetic determinant of the biosynthetic pathway for secondary metabolites was located within the 6.4-kb *Eco*RI fragment. It was also identified

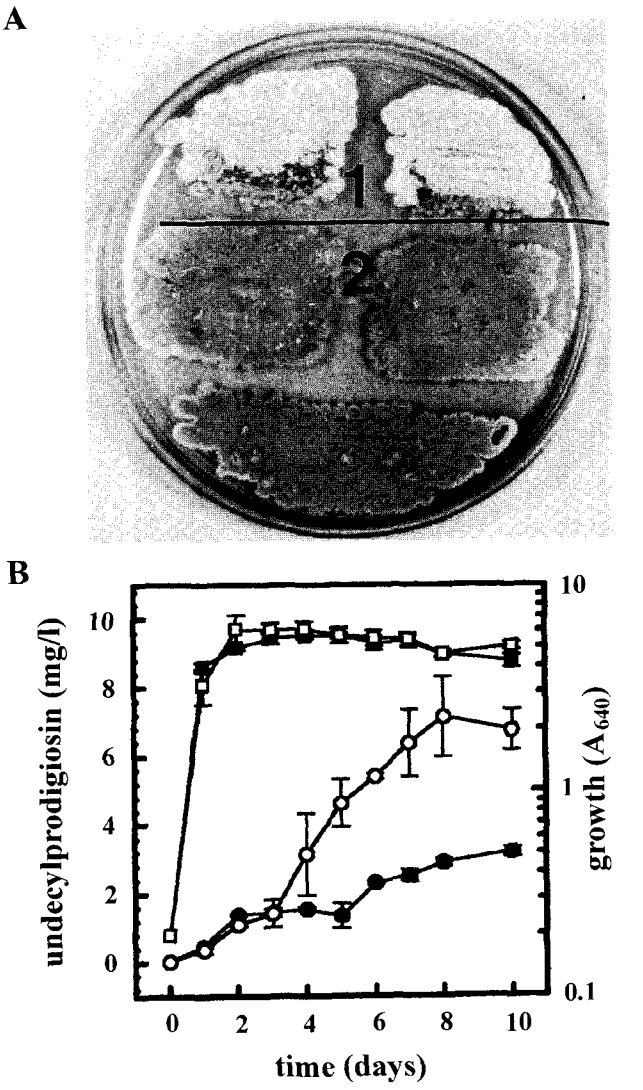

Fig. 1. Phenotypic characteristics relating to the production of secondary metabolites, which were induced by the introduction of pJWA2.

(**A**) *S. lividans* TK-24 transformed with pWHM3 (1) and pJWA2 (2) were grown on R2YE agar for 7 days for the visual inspection of pigment-development. (**B**) Growth and undecylprodigiosin production of *S. lividans* TK-24 transformed with pWHM3 or pJWA2 in R2YE broth. ○ and □ indicate undecylprodigiosin production and growth, respectively. The filled and unfilled symbols indicate *S. lividans* TK-24 with pWHM3 and pJWA2, respectively. The cultures were prepared with the inoculum of spores and mycelium from a culture plate 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 of 250 rpm.

that the 1.3-kb *Kpn*I fragment hybridized with pl-α3. A nucleotide sequence analysis of the 1.3-kb *Kpn*I region revealed 80-bp of the DNA sequence matched nearly perfectly with the cognate sequence within pl-α3. It is therefore plausible that the 80-bp DNA sequence is responsible for the detected signal hybridized with the pl-α3 probe. However, there was no homologous region in the 1.3-kb *Kpn*I fragment to coding regions found in the

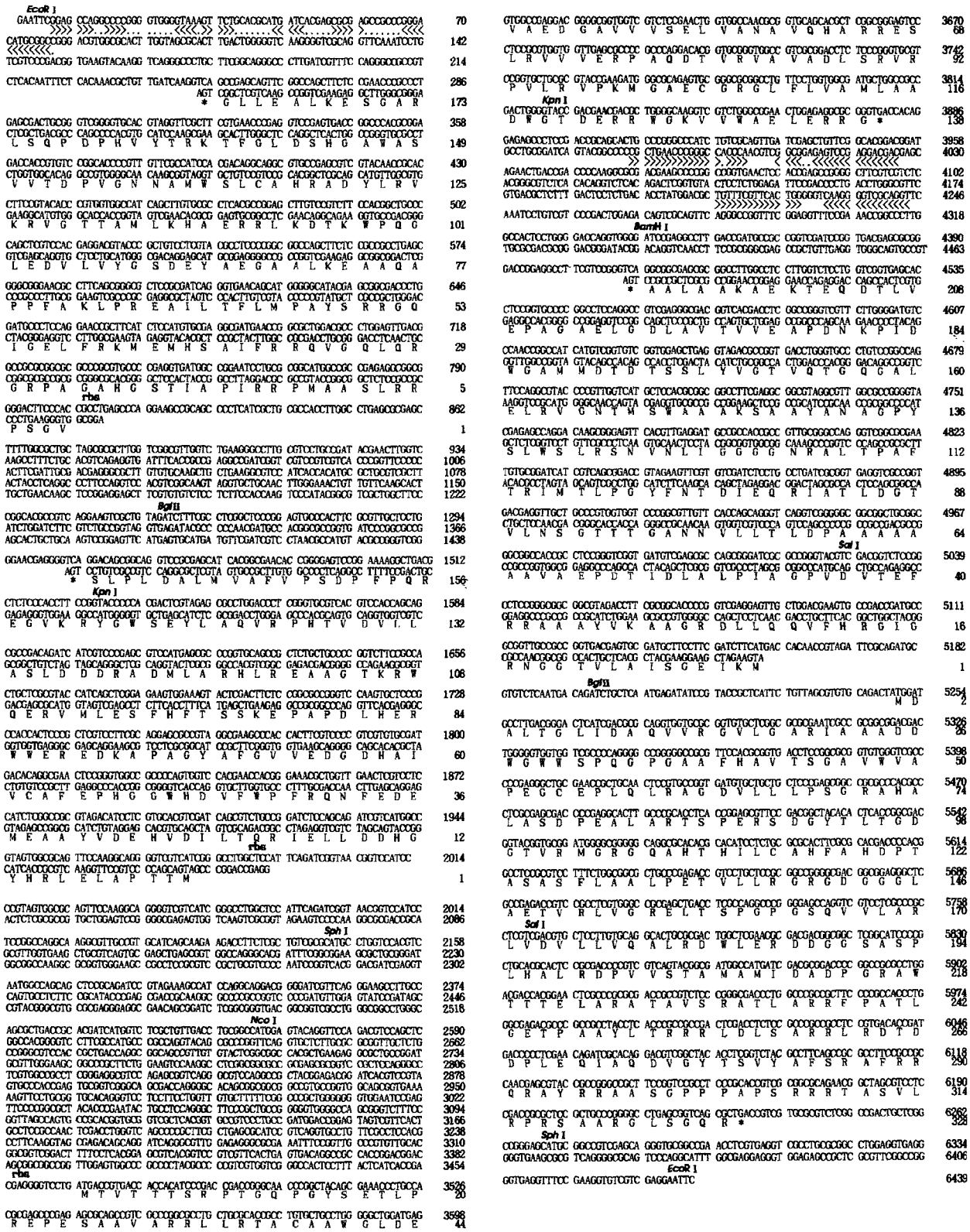

Fig. 2. Nucleotide sequence of the 6,439-bp *Eco*RI fragment from *Streptomyces albus* ATCC 21838.

The deduced amino acid sequence of the proposed translation product is given below the nucleotide sequence. The asterisks denote the translation termination codons. For ORFs reading right to left, both strands are shown. Potential ribosomal binding sites (rbs) are noted, as are significant restriction endonuclease sites. The putative $tRNA^{pro}$ sequence [44] (nt: 79-151) and inverted complementary sequences (nt: 4053-4098, 4283-4316) are indicated above the sequence. The nucleotide sequence was deposited in the GenBank database under the accession number AF145724.

p1-α3 nor to any of the known proteins contributing to the observed activation of antibiotic production. Accordingly, the nucleotide sequence of the 6.4-kb *Eco*RI fragment was determined to identify the genetic determinant which was responsible for the activation of undecylprodigiosin production.

Nucleotide Sequence Analysis of the 6.4-kb *Eco*RI Fragment

A computer-aided FRAME analysis [5] of the nucleotide sequence of the 6.4-kb *Eco*RI fragment predicted a putative five complete coding sequences, ORF1 with 184 amino acids, ORF2 with 175 amino acids, ORF3 with 137 amino acids, ORF4 with 223 amino acids, and ORF5 with 327 amino acids (Fig. 2). For each coding sequence, the average G+C content for the third position was 90.8, 86.4, 85.5, 92.4, and 87.2 mol%, respectively. The distribution of GC pairs at each of the three positions within the codons was in good agreement with the characteristic of most *Streptomyces* genes. For ORF4 and ORF5, the Shine-Dalgano sequence [18, 53] was not found in the expected positions. As in the case of the *aph* gene, there was no ribosomal binding site in front of some *Streptomyces* genes [56]. Other putative ORFs were discarded as coding regions because their codon usages did not fit the *Streptomyces* pattern. The imperfect inverted complementary repeat sequences at nt 4053 to 4098 ($\Delta G = -41.51$ kcal/mol) and nt 4283 to 4316 ($\Delta G = -37.60$ kcal/mol) may serve as transcriptional termination signals for ORF3 and ORF4, respectively.

ORF1 and ORF2 Were Predicted to Encode Proteins for Integrating Activity

The deduced amino acid sequence of ORF1 resembled the integrases/recombinases which are found in integrating conjugative plasmids or temperate bacteriophages (Fig. 3A). Integrases are known to show a large diversity of sequences while only exhibiting regional similarities, mostly in the 40-residues region near the C-terminus of the proteins [1]. The product of ORF1 also showed a high similarity in the C-terminus region to a known integrase/recombinase, and the alignment of the sequences revealed the conserved sequence of [D-X-RH-X_9-GVP-X_8-G-X_{10}-Y] at residues 129 to 164. The tyrosine residue at the end of this sequence may contribute to the active site of integrases [7]. Tyrosine, arginine, and histidine at the respective alignment positions of 164, 131, and 128 are highly conserved within the family of integrases [7]. Arg-131 and Tyr-164 were found correctly positioned in the predicted ORF1 protein, however, a tyrosine residue was found in place of His-128.

Integrases promote conservative site-specific recombination. Integrase genes are closely linked to the plasmid attachment site (*attP*) and $tRNA^{pro}$ gene that may serve as a chromosomal attachment site (*attB*) [44, 45]. The putative $tRNA^{pro}$ (nt 79 to 151) gene (GGG -anticodon at nt 34 to 36) was located downstream of ORF1, the sequence of which was also found in p1-α3 (data not shown). The deduced amino acid sequence of ORF2 showed a regional similarity to the KlcB protein found in the broad-host-range plasmid RK2 (31), with an identity score of 39% and a similarity of 48% between the residues 116 to 173 (data not shown). Accordingly, it was plausible that a fragment of the *S. albus* integrating element was hybridized with p1-α3 in the region of the $tRNA^{pro}$ sequence, which resulted in the isolation of the 6.4-kb *Eco*RI fragment conveying the genetic determinant(s) for antibiotic production linked to this putative integrating element.

The Products of ORF3, ORF4, and ORF5 Were Predicted to Be Responsible for the Activation of Antibiotic Production

A computer-aided search revealed that the product of ORF3 resembled the putative regulatory proteins of *S. coelicolor* such as *abaA*-ORFA [15] (Fig. 3B). The ORF3 product also showed a similarity to the RsbW of the *sigB* operon [26] and to the signal-transducing histidine kinase of *Archaeoglobus fulgidus* [29] (Fig. 3B). The *abaA* locus have been reported to enhance the production of actinorhodin in *S. lividans* and *S. coelicolor* when introduced on a multicopy plasmid [15]. However, the function of the ORFA protein in *abaA* has not yet been determined. RsbW is found in the *sigB* operon of gram-positive bacteria and controls gene expression at the transcriptional level for 'the general stress response' through protein-protein interaction [3, 58].

ORF4 and ORF5 appeared to form a unit that was divergently transcribed from a putative common promoter region in which the G+C contents of all the three frames were relatively low. A low G+C content is typical for putative promoter-containing regions in the *Streptomyces* species. The amino acid sequence of the ORF4 product was similar to that of a group of oxidoreductases (Fig 3C); β-ketoacyl reductase of *Vibrio harveyi* [52], aklaviketone reductase of *Streptomyces* sp. C5 [13], clavulanate-9-aldehyde reductase of *Streptomyces clavuligerus* [42], and retinal short-chain dehydrogenase of *Mus musculus* [20]. Although the putative ORF4 product is likely an oxidoreductase/dehydrogenase, its function is not clear. The amino acid sequence of the ORF5 product was similar to that of a group of bacterial transcriptional regulators of the AraC (arabinose operon regulator) family [17] (Fig. 3D); a putative AraC-like transcriptional regulator from Tn*4811* of *S. lividans* 66 [10], AlkR, a transcriptional regulator for the alkane hydroxylase of *Acinetobacter* sp. strain ADP1 [43], ArgR, the arginine regulatory protein of *Pseudomonas aeruginosa* [40], and SoxS, a transcriptional regulator of superoxide stress genes in *E. coli* [59].

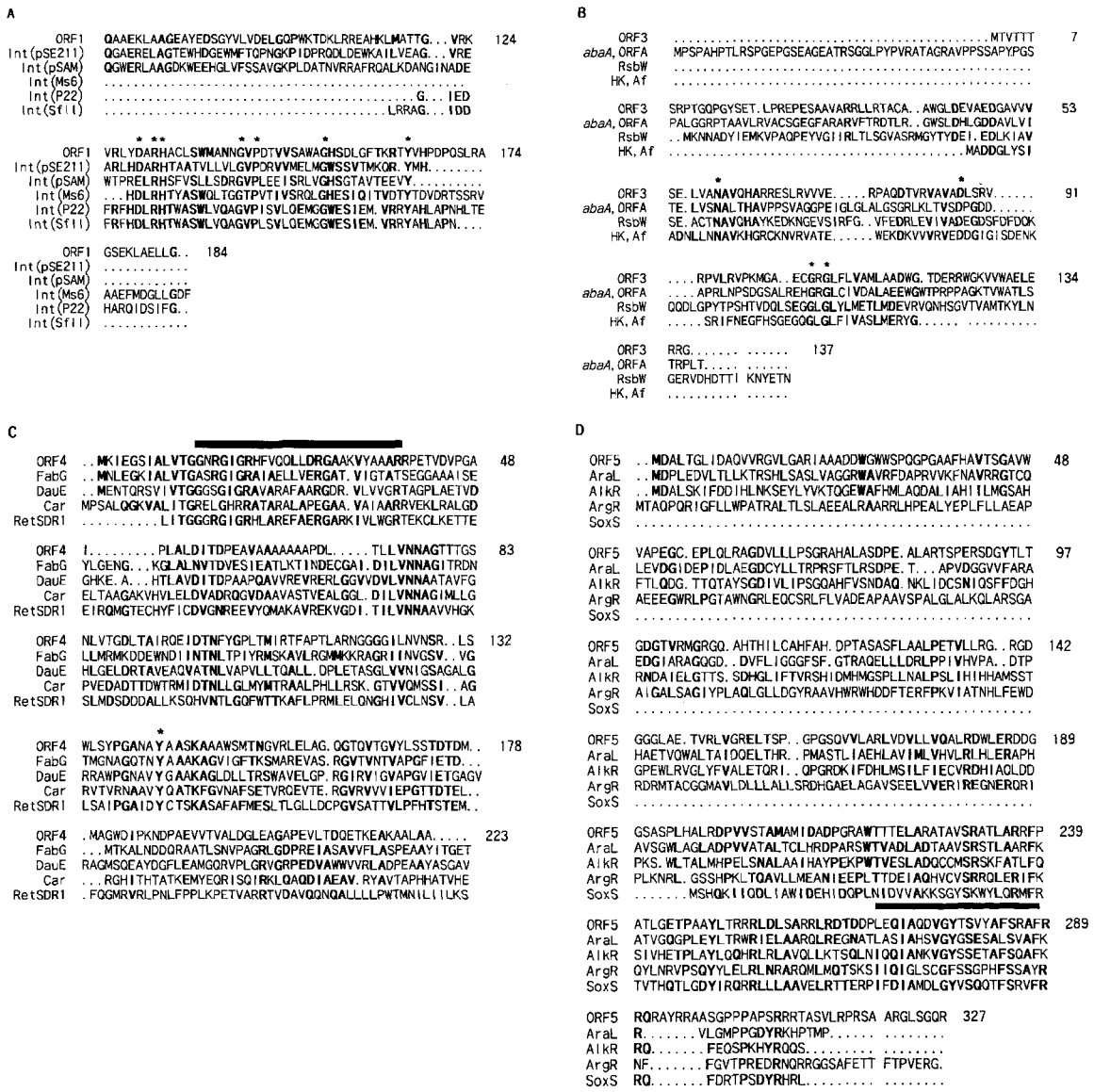

Fig. 3. Alignments of the deduced amino acid sequences of ORF1, 3, 4, and 5 with known proteins.

The amino acid sequences of ORF1, 3, 4, and 5 are always shown on top. The bold characters denote homologous amino acid residues. (**A**) Similarity of the ORF1 product (residues 78 to 184) to integrases (recombinases). The protein product of ORF1 was aligned with the amino acid sequences of the recombinase of plasmid pSE211 in *Saccharopolyspora erythraea* (residues 329 to 416) [8], integrase of pSAM2 in *Streptomyces ambofaciens* (residues 314 to 370) [7], integrase of *Mycobaterium* phage Ms6 (residues 274 to 363) [16], integrase of bacteriophage P22 (residues 307 to 366) [34], and integrase of bacteriophage SfII (residues 121 to 183) [37]. The numbers denote the amino acid residues of the ORF1 product. The asterisks above the alignment indicate the highly conserved amino acid residues. (**B**) Similarity of the ORF3 product (residues 1 to 137) to putative regulatory proteins. The protein product of ORF3 was aligned with the amino acid sequences of the ORFA of *abaA* in *Streptomyces coelicolor* (residues 1 to 192) [15], anti-sigma factor (RsbW) of the *sigB* operon in *Bacillus subtilis* (residues 1 to 160) [26], and a putative histidine kinase of *Archaeoglobus fulgidus* (residues 229 to 310) [29]. The numbers denote the amino acid residues of the ORF3 product. The asterisks above the alignment indicate conserved amino acid residues which are thought to be important for ATP binding in RsbW and in histidine kinase [27]. (**C**) Similarity of the ORF4 product (residues 1 to 223) to oxidoreductases. The protein product of ORF4 was aligned with the amino acid sequences of β-ketoacyl reductase of *Vibrio harveyi* (residues 1 to 234) [52], aklaviketone reductase of *Streptomyces* sp. C5 (residues 1 to 241) [13], clavulanate-9-aldehyde reductase of *Streptomyces clavuligerus* (residues 1 to 237) [42], and retinal short chain dehydrogenase of *Mus musculus* (residues 42 to 275) [20]. The numbers denote the amino acid residues of the ORF4 product. The bar above the sequence indicates the residues for NADP-binding, and the asterisk denotes the active tyrosine residue [52]. FabG is the most similar to the ORF4 product, with an identity score of 31% and a similarity of 45% using the Blast program at the NCBI [2]. (**D**) Similarity of the ORF5 product (residues 1 to 327) to the AraC family of transcriptional regulators. The protein product of ORF5 was aligned with the amino acid sequences of the putative AraC-like transcriptional regulator of Tn*4811* in *S. lividans* 66 (residues 1 to 304) [10]; AraL, AraC-like transcriptional regulator for the alkane hydroxylase of *Acinetobacter* sp. strain ADP1 (residues 1 to 306) [43]; ArgR, the arginine regulatory protein of *Pseudomonas aeruginosa* (residues 1 to 329) [40]; and SoxS, an activator of the superoxide stress genes in *E. coli* (residues 1 to 107) [59]. The numbers denote the amino acid residues of the ORF5 product. The bar above the sequence indicates the predicted helix-turn-helix DNA-binding domains [10]. AraL is the most similar to the ORF5 product, with an identity score of 35% and a similarity of 47% using the Blast program at the NCBI [2].

Phenotypic Characterization of the Antibiotic Production

To examine the effect on antibiotic production, ORF3, ORF4, and ORF5 were overexpressed separately using a constitutive *ermE* promoter on pWHM3 in *S. lividans* TK-24 (Fig. 4), and the resulting productivities of actinorhodin and undecylprodigiosin were investigated; pJHS3044 for the expression of the ORF3 product, pJHS3045 for the ORF4 product, pJHS3051 for the ORF5 product. When the undecylprodigiosin production was examined in the R2YE broth culture (Table 2), *S. lividans* TK-24 transformed with pJHS3045 and pJHS3051 showed a remarkable overproduction. This indicated that the ORF4 product and ORF5 product were contributive at least in part, to the activation of antibiotic production due to the introduction of pJWA2. *S. lividans* TK-24 transformed with pJHS3044 showed an increased productivity to the extent comparable to that of the strain with pJWA2, which indicated that the antibiotic productivity was not dependent on the intracellular number of the ORF3 product, at least in the R2YE broth culture. To determine whether or not the ORF3 product was related to the regulation of antibiotic production or if the effect of the ORF3 product depended on the culture media, the productivities of the transformants were investigated using an actinorhodin production medium.

For the selection of the actinorhodin production medium, different carbon sources and different phosphate concentrations were tested with regard to the pigment accumulation in the culture broth, following the recipe of Liao *et al.* [35]. Among those tested, the medium supplemented with glycerol as the carbon source and 0.6 mM phosphate appeared to be the most effective in actinorhodin production as well as undecylprodigiosin. The media composition may also render growth-associated actinorhodin production [35].

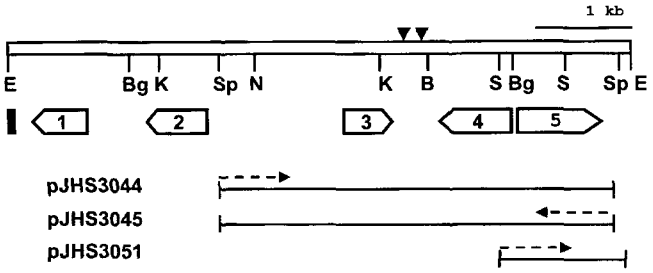

Fig. 4. Organization of genes found in the 6.4-kb *Eco*RI region.
The significant restriction endonuclease sites are noted below: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; K, *Kpn*I; N, *Nco*I; S, *Sal*I; Sp, *Sph*I. The inverted complementary sequences (nt: 4053-4098, 4283-4316) are indicated above the sequence as arrow heads. The putative $tRNA^{pro}$ sequence [44] (nt: 79-154) is presented along with the coding regions. The plasmids derived from pJWA2 are shown (Table 1) and the directions of the transcriptions from the *ermE* promoter [56] are indicated by dashed arrows.

Table 2. Undecylprodigiosin productivity of *S. lividans* TK-24 transformed with pWHM3 or its derivatives.

The strains of *S. lividans* TK-24	mg/g[a]
TK-24 (pWHM3 or pJHS14)	0.33±0.25
TK-24 (pJWA2)	1.43±0.2
TK-24 (pJHS3044)	1.40
TK-24 (pJHS3045)	2.50
TK-24 (pJHS3051)	2.40±0.40

[a]mg of undecylprodigiosin/g of dried cell weight.
The cultures were prepared in an R2YE broth with the inoculum of spores and mycelium from a culture plate of an R2YE agar. The culture was maintained in 50 ml of the broth in a 250-ml baffled flask at 28°C with a shaking speed of 250 rpm. The culture was maintained for 8 days and subjected to an assay for undecylprodigiosin production. To determine the dried cell weight (DCW), the mycelium was harvested by centrifugation and dried at 70°C to a constant weight.

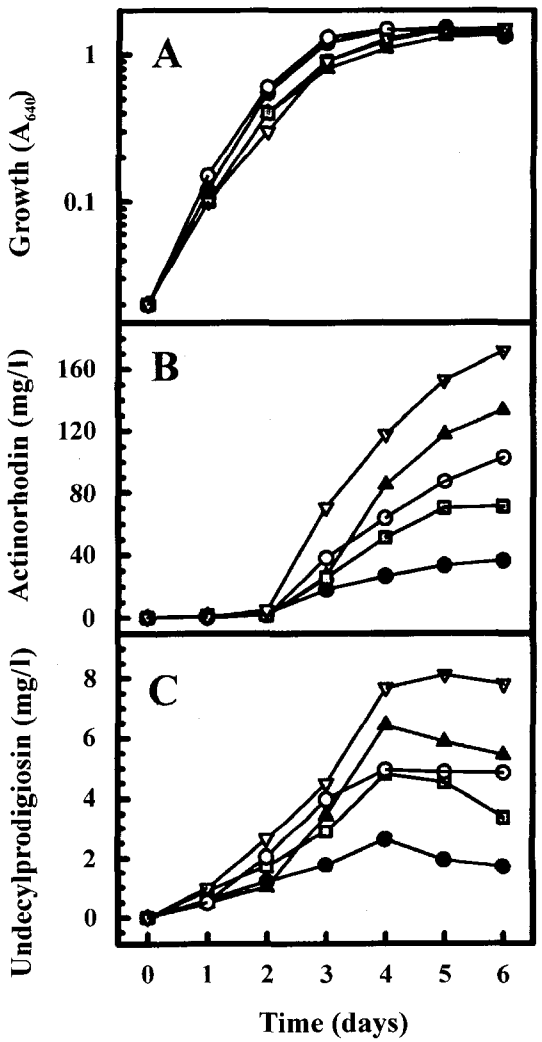

Fig. 5. Growth (**A**) and production of actinorhodin (**B**) and undecylprodigiosin (**C**) of *S. lividans* TK-24 transformed with pJHS14 or its derivatives in an actinorhodin production medium.
●, ○, ▲, □, and ▽ indicate the strains with pJHS14, pJWA2, pJHS3044, pJHS3045, and pJHS3051, respectively. The cultures were prepared in the production medium with the inoculum of the mycelium and spores from a plate culture 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 of 250 rpm.

The culturing of an inoculum with spores and mycelium from a culture plate showed that the strain with pJHS3051 yielded a remarkable overproduction of both actinorhodin and undecylprodigiosin (Fig. 5). Antibiotic production was induced upon entering the stationary phase in the strain with pJHS3044 and the productivity was higher than that of the strain with pJWA2 at 6 days after the initiation of the cultures (Fig. 5). A plausible explanation is that the activation of antibiotic production due to the ORF5 product is held by the high titre of the ORF3 product before reaching the stationary phase in the strain with pJHS3044.

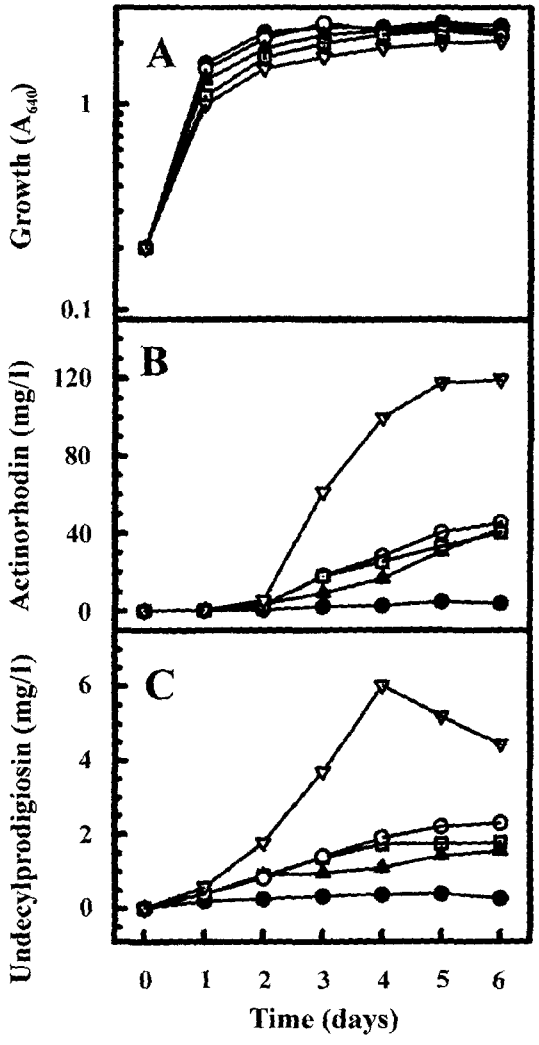

Fig. 6. Growth (**A**) and production of actinorhodin (**B**) and undecylprodigiosin (**C**) of *S. lividans* TK-24 transformed with pJHS14 or its derivatives in an actinorhodin production medium. ●, ○, ▲, □, and ▽ indicate the strains with pJHS14, pJWA2, pJHS3044, pJHS3045, and pJHS3051, respectively. 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 of 250 rpm and used to inoculate the production medium at 2% (v/v). The inoculated production mediums were maintained under the same culturing condition.

The production medium was inoculated with the mycelium of the R2YE broth culture and antibiotic production was determined (Fig. 6). *S. lividans* TK-24 transformed with pJHS3051 showed the highest productivity again, and both of the strains with pJHS3044 and pJHS3045 yielded productivities lower than one third of those of the strain with pJHS3051, with a negligible production of the strain with pJHS14 (Fig. 6). The strain with pJWA2 showed a productivity comparable to that of the strain with pJHS3044 or pJHS3045. With the unknown function of the ORF4 product, the AraC-like transcriptional regulator homologue is assumed to be the major factor in antibiotic production among the genes found in the 6.4-kb *Eco*RI fragment.

The pathway-specific activator genes, *act*II-ORF4 for actinorhodin and *redD* for undecylprodigiosin, were identified and their products were required for the expression of biosynthetic genes of actinorhodin and undecylprodigiosin, respectively [19, 55]. Thus, further works could be aimed for elucidating the relationship between the AraC-like transcriptional regulator homologue and the pathway-specific regulators.

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