

Expression of the *melC* Operon in Several *Streptomyces* Strains Is Positively Regulated by AdpA, an AraC Family Transcriptional Regulator Involved in Morphological Development in *Streptomyces coelicolor*

Dongqing Zhu, Xinyi He, Xiufen Zhou, and Zixin Deng*

Bio-X Life Science Research Centre and School of Life Science & Biotechnology,
Shanghai Jiaotong University, Shanghai, People's Republic of China

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Dark brown haloes of melanin around colonies are an easily visualized phenotype displayed by many *Streptomyces* strains harboring plasmid pIJ702 carrying the *melC* operon of *Streptomyces antibioticus* IMRU3270. Spontaneous melanin-negative mutants of pIJ702 occur with a frequency of ca. 1%, and often mutation occurs in the *melC* operon, which removes the BglII site as part of an inverted repeat. Other melanin-negative mutations seem to occur spontaneously in *Streptomyces lividans*, resulting in white colonies from which intact, melanin-producing pIJ702 can be isolated by introduction into a new host. *S. lividans* ZX66 was found to be such a mutant and to have a secondary mutation influencing expression of the *melC* operon on the chromosome. A 3.3-kb DNA fragment was isolated from its progenitor strain, JT46, and a gene able to restore *melC* operon expression was found to encode a member of an AraC family of transcriptional regulators, which was equivalent to AdpA_c in *Streptomyces coelicolor* and therefore was designated AdpA_l. Lack of *melC* operon expression was correlated with a single A-to-C transversion, which altered a single key amino acid residue from Thr to Pro. The transcription of the *melC* operon was found to be greatly reduced in the *adpA* mutant background. The counterpart gene (*adpA_l*) in the *S. antibioticus* strain in which the *melC* operon carried on pIJ702 originated was also isolated and was found to have an identical regulatory role. Thus, we concluded that the *melC* operon is under general direct positive control by AdpA family proteins, perhaps at the transcriptional level and certainly at the translational level via *bldA*, in *Streptomyces*.

Tyrosinase (EC 1.14.18.1) is a ubiquitous copper-containing monooxygenase that catalyzes both the O hydroxylation of monophenols and the oxidation of *O*-diphenols to *O*-quinones (29, 32) responsible for the biosynthesis of melanin pigment from tyrosine (29) in bacteria. In *Streptomyces antibioticus* (23) and *Streptomyces glaucescens* (16), the tyrosinase gene (*melC2*) is preceded by the *melC1* gene encoding a conserved protein essential for the expression of melanin (3, 23, 28) in a polycistronic operon. The MelC1 protein, which behaves like a molecular chaperone, has dual roles; it regulates copper incorporation, and it promotes secretion of apotyrosinase via a transient MelC1-MelC2 complex (7, 8, 28, 30).

The *Streptomyces melC* operon has been used extensively as a phenotypic marker for *Streptomyces* plasmids (23) and for the construction of promoter-probe vectors for *Streptomyces* (36) and *Escherichia coli* (9). Investigation of the control of the *melC* operon revealed that a cloned gene of *Streptomyces lividans* 66, *cutR*, phenotypically suppresses defective *melC1* (44). The CutR protein resembles the response regulator OmpR of the osmoregulatory signal transduction system in *E. coli*. Next to *cutR* is *cutS*, which encodes the histidine protein kinase counterpart of OmpS. Thus, the putative *cutR-cutS* operon was postulated to regulate copper metabolism in *Streptomyces* (44).

In *Rhizobium leguminosarum* biovar phaseoli, transcription of a gene needed for melanin synthesis was found to be activated by *nifA* of *Rhizobium* and *Klebsiella pneumoniae* (15), which is an activator of *nifH* of *K. pneumoniae* in *E. coli* cells grown with low oxygen concentrations. Analysis of *Streptomyces griseus* mutants defective in melanogenesis showed that there was a close regulatory correlation between melanogenesis and morphological and physiological differentiation. Also, introduction of plasmids carrying the *melC* operon failed to confer melanin production in the melanin-negative mutants, and disruption of *melC2* barely affected melanin productivity, suggesting that another unknown enzyme is involved in melanogenesis in *S. griseus* (11).

Here we demonstrate unambiguously that expression of the *melC* operon in *S. lividans* and *Streptomyces coelicolor* and in its native host, *S. antibioticus*, is under strict positive control by a gene encoding a multifunctional regulatory protein (AdpA_l). A counterpart protein in *S. coelicolor* (AdpA_c) was shown to be the product of the *bldH* gene (47), which contains a rare leucine codon (TTA) controllable by *bldA* tRNA at the translational level, and to be closely associated with aerial mycelium development (41).

MATERIALS AND METHODS

Bacterial strains and plasmids. *Streptomyces* and *E. coli* strains used in this study are listed in Table 1.

General methods and techniques. The general growth media and conditions used for *E. coli* and *Streptomyces* strains and the standard methods used for handling *E. coli* and *Streptomyces* in vivo and in vitro, such as preparation of

* Corresponding author. Mailing address: Bio-X Life Science Research Center, Shanghai Jiaotong University, Shanghai 200030, People's Republic of China. Phone: 86 21 62933404. Fax: 86 21 62932418. E-mail: zxdeng@sjtu.edu.cn.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant phenotype and/or characteristics ^a	Reference or source
<i>S. coelicolor</i> A3(2) strains		
M145	Wild type	18
ZD2	M145-derived <i>adpA_c</i> disruptant, <i>aac(3)IV</i>	This study
<i>S. lividans</i> 66 strains		
1326	Wild type, Dnd ⁺ /φHAU3 ^r	31
JT46	Dnd ⁺ /φHAU3 ^r	43
ZX64	JT46-derived <i>dndA</i> disruptant, Dnd ⁻ /φHAU3 ^r	Zhou, unpublished data
HXY1	JT46-derived <i>dndA</i> disruptant, Dnd ⁻ /φHAU3 ^r	He, unpublished data
ZX66	Same as JT46 but <i>adpA₁</i>	This study
ZD1	ZX66 derivative with an autonomous pIJ702 but integrative, pJTU1452, <i>aac(3)IV</i> /Mel ⁺	This study
ZD3	JT46-derived <i>adpA₁</i> disruptant, <i>aac(3)IV</i>	This study
<i>S. antibioticus</i> strains		
IMRU3720	Wild type, Mel ⁺	
ZD4	IMRU3720-derived <i>adpA_c</i> disruptant, <i>aac(3)IV</i>	
<i>E. coli</i> strains		
DH10B	<i>recA</i>	GIBCO BRL
ET12567/pUZ8002	<i>recE dam dcm hsdS</i> Cm ^r Str ^r Tet ^r Km ^r	35
Plasmids		
pIJ702	pIJ101 derivative, <i>tsr mel</i>	23
pSET152	<i>aac(3)IV lacZ rep^{pucc} att^{bc31} oriT</i>	5
pOJ260	<i>apr oriT rep^{pucc} lacZ</i>	26
pMD18-T	pUC18 derivative	TaKaRa
pJTU1452	ca. 3.5-kb PCR fragment (obtained by using pSET152-FP and pSET152-RP as primers and ZD1 DNA as the template) inserted into the EcoRV site of pSET152	This study ^b
pJTU1454	Religation of pJTU1452 after digestion with BamHI and Bg/II, AdpA ₁ ⁺	This study ^b
pJTU1455	Religation of pJTU1452 after partial digestion with ApaI, AdpA ₁ ⁻	This study ^b
pJTU1457	Religation of pJTU1452 after digestion with ApaI, AdpA ₁ ⁺	This study ^b
pJTU1458	921-bp AluI fragment after PCR amplification (obtained by using <i>sgb118</i> and <i>sgb218</i> as primers and M145 DNA as the template) inserted into the EcoRV site of pOJ260	This study
pJTU1459	921-bp AluI fragment from pJTU1452 inserted into the EcoRV site of pOJ260	This study
pJTU1461	ApaI fragment (1.7 kb) of ZX66 containing complete <i>adpA₁</i> * inserted into the ApaI site of Bluescript SK (+)	This study
pJTU1465	ApaI fragment (1.5 kb) of IMRU3720 containing complete <i>adpA_a</i> inserted into the ApaI site of Bluescript SK(+)	This study
pJTU1466	958-bp PCR fragment (obtained by using A1296 and pSET152-FP as primers and pJTU1465 as the template) internal to <i>adpA_a</i> inserted into the EcoRV site of the pMD18-T vector	This study
pJTU1467	HindIII-EcoRI fragment carrying, the 958-bp PCR fragment of pJTU1466 inserted into the corresponding sites of pOJ260	This study

^a *mel*, tyrosinase gene for melanin production; *oriT*, origin of transfer of plasmid RK2; *tsr*, thiostrepton resistance gene; *aac(3)IV*, apramycin resistance gene; Cm^r, chloramphenicol resistance.

^b See Fig. 2.

plasmid and chromosome DNAs, transformation, etc., were those described previously (26, 38), unless indicated otherwise. *E. coli* strains were cultivated at 37°C in Luria-Bertani medium or on Luria-Bertani agar plates. *Streptomyces* strains were routinely grown at 30°C on SFM solid medium (17) for conjugation between *E. coli* and *Streptomyces*, on R2YE (42) for protoplast transformation, on MMT for tyrosinase expression and melanin production, or in YEME and TSBY liquid media containing 34% sucrose for mycelial growth. Plasmid and total DNAs were isolated from *Streptomyces* strains as described by Kieser et al. (26). Unmethylated DNA was prepared from *E. coli* ET12567. In vivo generation of targeted mutations in *Streptomyces* was achieved by conjugation between *E. coli* and *Streptomyces* strains as described by Sun et al. (40). DNA sequencing was carried out by using ABI Prism BigDye terminator cycle sequencing Ready Reaction kits from PE Applied Biosystems. DNA fragments were subcloned into pBluescriptII SK(+), and this was followed by unidirectional subcloning with the Erase-a-Base system (Promega). The double-stranded plasmid DNA used as the template was purified by polyethylene glycol 8000 precipitation before sequencing. About 200 to 400 ng of template DNA was mixed with 4 μl of a terminator Ready Reaction mixture and the appropriate primer, and the total volume was adjusted to 20 μl with deionized water. The sequencing reaction mixtures were subjected to cycle sequencing with a DNA thermal cycler (Perkin-Elmer) before the sequencing gel was examined with an ABI 377 autosequencer. The sequence

data were analyzed with the Frame-Plot online program (20). DNA and deduced protein sequence homology searches were performed by using BLAST (1, 2, 39) and FASTA (37). Multiple alignments of sequences were constructed by using the BioEdit sequence alignment editor (14). The copy numbers of plasmids were estimated by densitometric scanning of band intensity after agarose gel electrophoresis as described by Kieser et al. (25). Preparations that were compared were always run on the same gel. For *Streptomyces*, apramycin and thiostrepton were used at concentrations of 30 and 10 μg ml⁻¹, respectively, in SFM agar and at concentrations of 15 and 5 μg ml⁻¹, respectively, in liquid media.

Cloning of a DNA fragment restoring the melanin phenotype to ZX66 in *S. lividans*. *S. lividans* JT46 total DNA was partially digested with MboI to obtain a fragment size of 2 to 8 kb, treated with alkaline phosphatase to minimize formation of multiple inserts, and ligated with BamHI-digested DNA of an integrative vector, pSET152 (26). The ligation mixtures were used to transform ZX66/pIJ702 protoplasts, and colonies with restored production of black pigment were selected. The DNA insert obtained from one black colony (ZD1) was recovered by using a pair of PCR primers (pSET152-FP [5'-CCAGTCACGAC GTTGTAAAACGA-3'] and pSET152-RP [5'-ACAGGAAACAGCTATGACA TGAT-3']) flanking the multiple cloning sites of pSET152 and chromosomal DNA of ZD1 as the template.

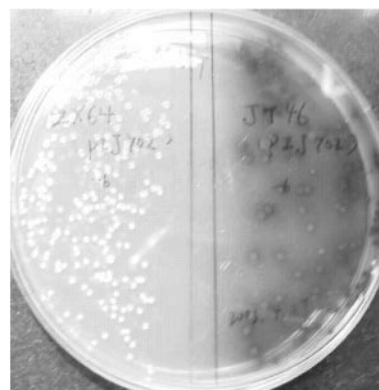
PCR primers and conditions and Southern and colony hybridizations. Oligonucleotide primers pSET152-FP and pSET152-RP were used to recover by PCR amplification a ca. 3.5-kb DNA fragment that included *adpA₁* cloned and integrated into the ZD1 chromosome before it was inserted into the EcoRV site of pSET152 to obtain pJTU1452. The primers were synthesized commercially. PCRs were performed by using a HYBAID PCR machine. A typical PCR mixture (50 μ l) consisted of 1.5 mM MgCl₂, 5 ng of genomic DNA as the template, 5% dimethyl sulfoxide, each deoxynucleoside triphosphate at a concentration of 100 μ M, and 25 pmol of the required primers in 1 \times PCR buffer. After addition of 5 U of *Pfu* polymerase (13), the DNA template was denatured at 95°C for 3 min. Amplification was carried out by 30 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 55°C, and extension for 4 min at 72°C, followed by extension for 5 min at 72°C. PCR products were then purified from 0.8% agarose gels by using a Gene Recovery kit (12). For Southern hybridization experiments, DNA was cleaved with restriction enzymes, separated in 0.8% agarose gels, and transferred onto a Hybond-N⁺ nylon membrane (Amersham-Pharmacia). α -[³²P]dCTP-labeled radioactive probes and a Random Priming kit (Roche) were used for both Southern blotting and in situ colony hybridization.

Cloning of *adpA from *S. lividans* ZX66 and *adpA_a* from *S. antibioticus* IMRU3720.** Total DNAs of ZX66 and IMRU3720 were digested with *Apa*I (a 1.5-kb *Apa*I fragment in IMRU3720 DNA was found to contain *adpA_a* by Southern hybridization), and ca. 1.5- to 1.7-kb fragments were recovered from an agarose gel for cloning into *Apa*I-digested and calf intestinal alkaline phosphatase-treated pBluescript SK(+). pJTU1461 was found to carry a 1.7-kb *Apa*I fragment of *adpA₁* from ZX66, and pJTU1465 carried a ca. 1.5-kb *Apa*I fragment of *adpA_a* from IMRU3720, as determined by colony hybridization in which a 1.7-kb *Apa*I fragment of *adpA₁* was used as the probe.

Constructs for generation of an *adpA₁* mutant of *S. lividans* JT46 (ZD2), an *adpA_c* mutant of *S. coelicolor* M145 (ZD3), and an *adpA_a* mutant of *S. antibioticus* IMRU3720 (ZD4) by targeted gene disruption. The pJTU1458 vector mediating disruption of *adpA_c* was constructed by PCR amplification of a ca. 3.3-kb DNA fragment covering *adpA_c* from the *S. coelicolor* M145 chromosome by using oligonucleotide primers 1458-FP (5'-GACAAGCCCCGCAACCTC-3') and 1458-RP (5'-GGCCTCGTCTCAAACGC-3'), followed by digestion with *Alu*I to generate a 921-bp internal fragment for insertion into the EcoRV site of *E. coli* plasmid pOJ260. Similarly, vector pJTU1459 mediating disruption of *adpA_c* was constructed by recovery of a 921-bp DNA fragment after *Alu*I digestion of pJTU1452 for insertion into the EcoRV site of pOJ260. Vector pJTU1467 mediating disruption of *adpA_a* was constructed by cloning a 958-bp PCR fragment covering part of *adpA_a* from pJTU1465 by using oligonucleotide primers pSET152-FP (5'-CCAGTCACGACGCTTGATAAACGA-3') and A1296 (5'-GAGATCCATACGCTGTC-3') into the EcoRV site of the pMD18-T vector to produce pJTU1466, from which an *Eco*RI-*Hind*III fragment was recovered for insertion into the corresponding site of pOJ260 to obtain pJTU1467.

Reverse PCR for transcript analysis. Total RNAs from JT46/pIJ702 and ZD3/pIJ702 were isolated by using an RNeasy mini kit (QIAGEN) and were treated with DNase. Reverse transcription RCR (RT-PCR) was performed by using a OneStep RT-PCR kit and Q-Solution (QIAGEN). The cycling parameters used were 30 min at 50°C and 15 min at 95°C for the reverse transcription step, followed by 30 PCR cycles of amplification (30 s at 94°C, 30 s at 55°C, and 30 s at 72°C). Primers melF (5'-ATGCGGGGTCGTCAC-3') and melMR (5'-CGTAGTGGCTGACGACGCTGA-3') were used as the oligonucleotide primers for RT-PCR experiments to amplify a 395-bp fragment internal to *melC*.

Expression of AdpA₁ in *E. coli* BL21(DE3), preparation of total proteins with or without AdpA, and gel mobility shift assay. The complete *adpA₁* sequence was amplified by PCR by using pJTU1457 DNA as the template DNA and oligonucleotide primers 1464F-*Nde*I (5'-GGGGGGCTTAGCCATATGAG-3') and 1464R-*Xho*I (5'-GGAGCGTCTGCTCGAGTCAC-3'). The amplified fragment was digested with *Nde*I plus *Xho*I and cloned in pET15b to generate pJTU1464. The overproduced AdpA₁ could be detected as both soluble and insoluble forms, but we failed to obtain a sufficient amount of the pure His-tagged AdpA₁ protein using nickel columns; thus, total proteins were isolated for a gel mobility shift assay essentially by the method of Vujaklija et al. (46). In brief, 3,000 cpm of ³²P-labeled probe DNA was incubated with total proteins with or without AdpA (0.2 to 1 μ g) at 30°C for 30 min in 40 to 50 μ l (total volume) of a buffer containing 50 mM sodium phosphate buffer (pH 8.0), 10% glycerol, 1 μ g of poly(dI-dC)-poly(dI-dC), and 0.01% bovine serum albumin as a protein stabilizer. After incubation, complexes and free DNA were resolved on nondenaturing 4 or 6% polyacrylamide gels (3% acrylamide-0.8% bisacrylamide) with a running buffer containing 40 mM Tris-HCl (pH 7.8), 20 mM sodium acetate, and 1 mM EDTA. The gels were dried, and the results were detected with a phosphorimager (FUJIFILM FLA-3000). A 295-bp sequence containing the promoter region of *melC* (from position -261 to position 34 with respect to



ZX66/pIJ702 JT46/pIJ702

FIG. 1. *melC* operon is expressed in JT46 (right) but not in ZX66 (left). Both strains were transformed with pIJ702 carrying *melC1* and *melC2* and were grown on MMT containing thiostrepton.

the transcriptional start point) was prepared by PCR by using primers melupF (5'-GACCCGCAAACCCCTTGATCCGC-3') and melupR (5'-CCTCCTGGG GTGCGTTGGGTTGA-3'), and the 5' ends were labeled with [γ -³²P]ATP and T4 polynucleotide kinase.

RESULTS

ZX66, an *S. lividans* ZX64 derivative incapable of expressing the *melC* operon. ZX64 was a mutant that was generated from *S. lividans* JT46 by insertion of a spectinomycin-streptomycin resistance cassette (*spc-str*) into *dnaA*, one of the five open reading frames (ORFs) in the *dnd* gene cluster (which contained genes that rendered DNA susceptible to degradation during electrophoresis [the Dnd phenotype] [X. Zhou and Z. Deng, unpublished]). When pIJ702 carrying the melanin (*melC*) operon consisting of *melC1* and *melC2* from *S. antibioticus* (23) was introduced into *S. lividans* ZX64 by protoplast transformation, however, mostly black colonies and occasionally a rare white colony (ZX66) were obtained, while all JT46 transformants were black. The inability of ZX66 to produce melanin was found to be stable (Fig. 1), and the sporulation phenotype of this strain seemed to be poorer than that of JT46.

Lack of expression of the *melC* operon is not due to *melC* mutation and could be restored by a 3.5-kb DNA fragment from JT46, a progenitor of ZX66. The copy number of pIJ702 seemed to be unchanged, and pIJ702 did not seem to have undergone any rearrangement because its banding patterns remained the same as those of pIJ702 present in the starting strain (JT46). The *MelC1* and *MelC2* genes on pIJ702 did not seem to be mutated as the production of black pigment after pIJ702 was reisolated from ZX66 and reintroduced into JT46 was normal. A library with 2- to 8-kb DNA inserts generated by *Mbo*I partial digestion of JT46 total DNA was ligated with *Bam*HI-digested pSET152, a plasmid vector able to integrate into the *S. lividans* chromosome. When pSET152-derived ligation mixtures were used to transform ZX66/pIJ702 protoplasts, a black transformant (ZD1) was obtained.

The DNA insert of ZD1 was recovered by using a pair of PCR primers (pSET152-FP and pSET152-RP) flanking the multiple cloning sites of pSET152 and chromosomal DNA of

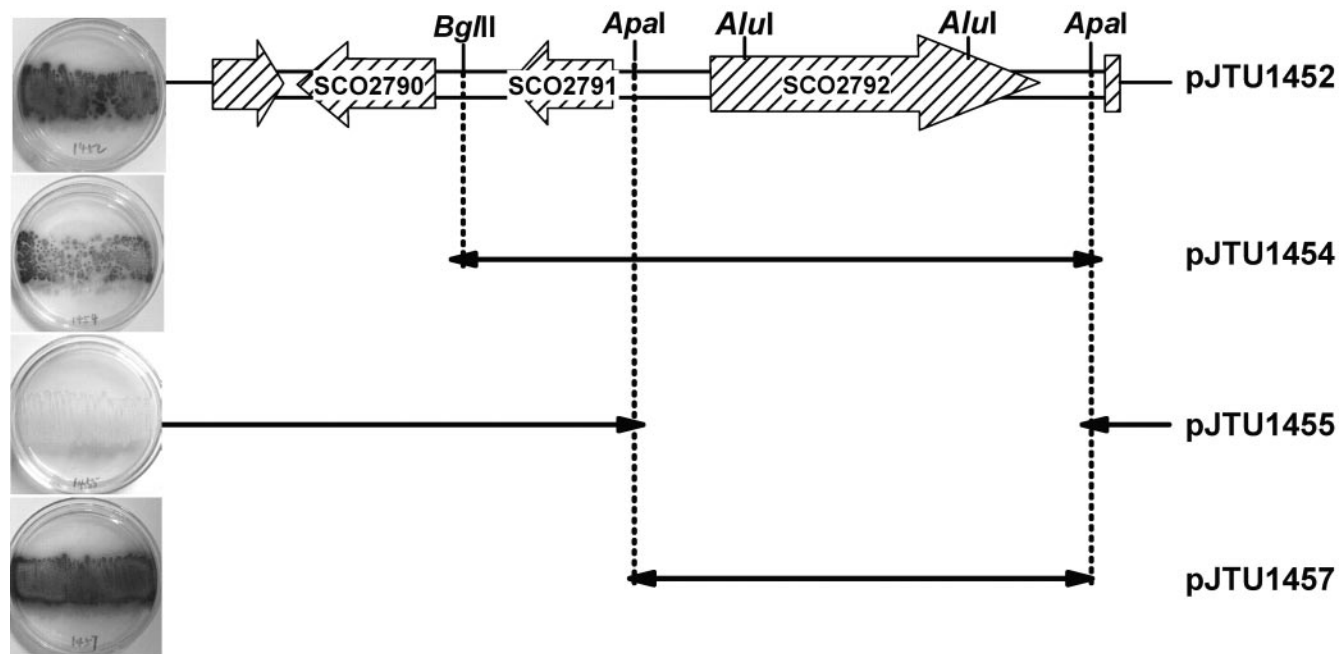


FIG. 2. Localization of a gene activating *melC* expression. The DNA sequence and genetic organization of the original 3,336-bp insert isolated from *S. lividans* in pJTU1452 are almost identical to the DNA sequence and genetic organization of the corresponding region carrying three complete ORFs (SCO2790 to SCO2792) in *S. coelicolor*, which is shown at the top. pJTU1452 derivatives are shown below pJTU1452, and their abilities to activate *melC* operon expression (black colonies on plates) are shown on the left.

ZD1 as the template. A 3.5-kb DNA fragment was amplified, and reinsertion of this fragment into pSET152 (resulting in pJTU1452) and introduction by transformation into ZX66 could complement full expression of the *melC* operon (data not shown).

Localization of a gene complementing *melC* expression deficiency. The 3.5-kb DNA insert from pJTU1452 was sequenced. BLAST searches immediately revealed strong similarity or even identity to a region (nucleotides 3045346 to 3048682) of the sequenced *S. coelicolor* genome which contained three complete ORFs, SCO2790, SCO2791, and SCO2792 (Fig. 2). Two incomplete ORFs (SCO2789 and SCO2793) (Fig. 2) seemed to be irrelevant to the control of *melC* expression and thus were not included in further analysis. One of the three complete ORFs (SCO2792; nucleotides 3047135 to 3048331) was clearly identified as an *araC* family transcriptional regulator, a potential candidate for controlling *melC* operon expression; SCO2790 showed significant homology to the USP domain of a family of universal *Streptomyces* proteins; and SCO2791 encoded a hypothetical protein without obvious conservation with other known proteins in the database.

To localize the responsible gene(s), religation of pJTU1452 after digestion with BamHI (flanking one side of the multiple cloning sites of the pSET152 vector) and BglII yielded pJTU1454; this removed SCO2790 (Fig. 2). In a similar way, religation of pJTU1452 after digestion with ApaI produced pJTU1455; this removed SCO2792 completely but left SCO2790 and SCO2791 intact (Fig. 2). Meanwhile, the 1.7-kb ApaI fragment carrying intact SCO2792 was inserted into the

corresponding site in pSET152, resulting in pJTU1457, which carried only SCO2792 (Fig. 2).

When the three pJTU1452 derivatives (pJTU1454, pJTU1455, and pJTU1457) were introduced by transformation into ZX66, black colonies were observed only when inserts carried SCO2792 alone (pJTU1457) or in combination with SCO2791 (Fig. 2). This experiment unambiguously demonstrated that SCO2792 is a required positive regulator of melanogenesis.

The locus that positively controls *melC* expression is equivalent to *adpA* (*adpA_I*) in *S. coelicolor*, and analysis of the *adpA_I* mutant allele in ZX66. The gene that positively controls *melC* operon expression in *S. lividans*, SCO2792, is an *adpA_c* homolog that has been reported to contain a rare leucine codon (TTA) whose translation depends on *bldA* tRNA and is involved in aerial mycelium development in *S. coelicolor* (41). We used the subscripts c and I, as used by Takano et al. (41), to distinguish between the genes of *S. coelicolor* and *S. lividans*, respectively. *adpA_c* in *S. coelicolor* and *adpA_I* in *S. lividans* are 99% identical at the nucleotide level, and four of the different nucleotides resulted in only one variation at the amino acid level. Similarly, the N-terminal portion of AdpA_I (and AdpA_c) has sequence homology with a group of proteins in the ThiJ-PfpI-DJ-1 family, which is widely distributed in bacteria, archaea, and eukaryotes, and has structural similarity to the type I glutamine amidotransferase domain (19), although the amino acid sequences of the members of this family were not dramatically conserved. This potential AraC family transcriptional regulator has a typical conserved DNA-binding domain with two helix-turn-helix motifs at the C terminus and a sequence

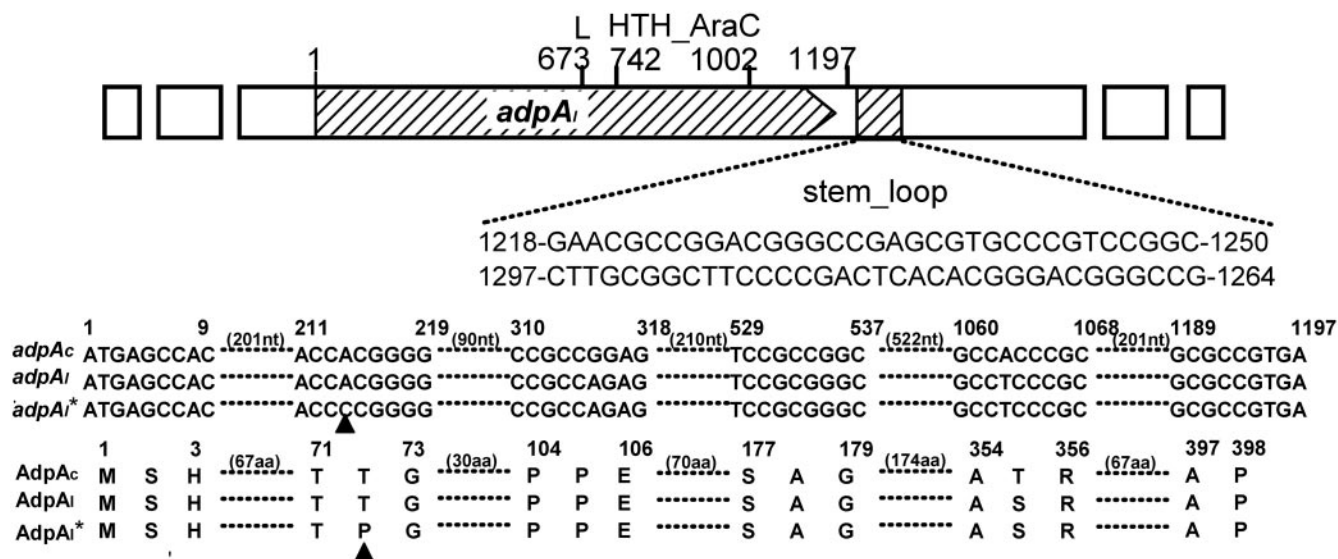


FIG. 3. Overall characteristics of the *adpA₁* gene and alignment of *adpA_c* (from *S. coelicolor* M145), *adpA₁* (from *S. lividans* ZX64), and *adpA₁** (from *S. lividans* ZX66) and the protein sequences encoded by these genes. Nucleotides are numbered from the ATG start codon. L is the rare leucine (TTA) codon. HTH_AraC is a region with a helix-turn-helix motif for the typical AraC family of bacterial regulatory proteins, and the shaded region (with two alternative sequences) has the potential to form a stem-loop structure. Only the regions with differences at the nucleotide (nt) and amino acid (aa) levels between alleles are shown, and mutant positions are indicated by triangles.

with the potential to form a stem-loop structure 21 bp downstream of the stop codon of AdpA_c or AdpA₁ (Fig. 3). A rare leucine codon (TTA) was found at the same position in both proteins (AdpA_c and AdpA₁) (Fig. 3), which must be the target for *bldA* regulation. When pIJ702 was introduced into an *S. coelicolor* M145-derived *bldA* mutant strain (J1700), none of the many independent thio streptomycin-resistant transformants produced a black pigment, as expected.

A 1.7-kb ApaI fragment carrying a hypothetical mutant *adpA₁* locus corresponding to SCO2792 in ZX66 was cloned into the corresponding site of pBluescript SK(+) by colony hybridization by using the 1.7-kb ApaI fragment carrying the JT46 *adpA₁* gene as a probe and sequenced. A transversion (A to C) was found at nucleotide 214, which changed a codon from ACG (for Thr) to CCG (for Pro) (Fig. 3). Indeed, alignment of the nucleotide (and amino acid) sequences of *adpA_c* and *adpA₁*, as well as other sequenced *adpA* genes (and the corresponding proteins) in *Streptomyces avermitilis* (*adpA_a*) and *S. griseus* (*adpA_g*), revealed that none of the *adpA* genes has any variation at nucleotide 214 (A is part of the ACG codon encoding Thr).

Expression of the *melC* operon is under the same positive control by AdpA_c in *S. coelicolor* M145 and by AdpA_a in its native host, *S. antibioticus*. pIJ702 was introduced into ZD2 (an M145 derivative with a mutation in *adpA_c*) and ZD3 (a JT46 derivative with a mutation in *adpA₁*). No black pigment was secreted from ZD2 or ZD3 carrying pIJ702 or from ZD4 (an *S. antibioticus* IMRU3720 derivative with a mutation in *adpA_a*), while a black pigment was clearly observed with M145 and JT46 carrying the same plasmid, as well as with the wild-type strain *S. antibioticus* IMRU3720 on the same plates (Fig. 4), suggesting that the *melC* operon is under the same positive control in all three organisms. Meanwhile, a sharp increase in the amount of black pigment was observed when an extra copy

of *adpA₁* was added by integrating pJTU1457 into the *attB* sites of either *S. coelicolor* or *S. lividans* strains carrying pIJ702 or *S. antibioticus* IMRU3720, from which the *melC* operon carried on pIJ702 originated (Fig. 4), by site-specific recombination through the *attP* site of ϕ C31 on pSET152.

AdpA₁ affects colony phenotypes and transcriptionally interacts with the promoter region of the *melC* operon. Constructed gene disruptants with disruptions internal to *adpA_c* in M145 (ZD2), *adpA₁* in JT46 (ZD3), and *adpA_a* in *S. antibioticus* IMRU3720 (ZD4) were compared in order to examine their colony phenotypes along with those of the corresponding parental strains. All of the mutants appeared to be obviously bald, as reported previously for the *bldA* mutant of *S. coelicolor* M145 (41), while all of the parental strains sporulated well.

An attempt was also made to detect the possible interaction of AdpA₁ with the promoter region of the *melC* operon by a gel mobility shift assay. As shown in Fig. 5, the total proteins isolated with AdpA₁ from *E. coli* strain BL21(DE3) carrying pJTU1464 (a pET15b derivative carrying the *adpA* gene) obviously bound to the 295-bp DNA fragment carrying the promoter region of the *melC* operon radioactively labeled with [γ -³²P]ATP and T4 polynucleotide kinase, while the total proteins without AdpA₁ from *E. coli* strain BL21(DE3) carrying pET15b lacked specific binding. Additionally, the band in lane 3, in which 0.02 μ g of total proteins was added, was obviously more intense than the band in lane 2, in which only 0.01 μ g of total proteins was added. The same experiments were repeated by using various concentrations of the same protein preparations with and without AdpA₁ but with an unrelated 318-bp labeled probe fragment as an additional control, and no shifted band, like that in lanes 2 and 3, was detected on the gel (data not shown).

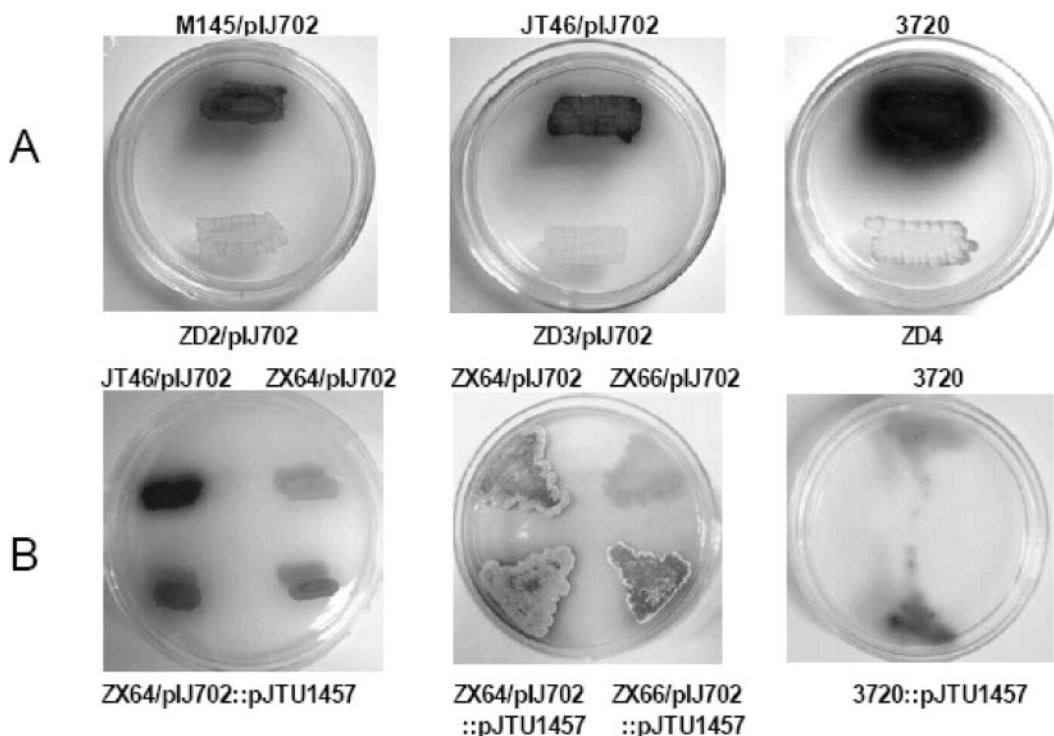


FIG. 4. (A) Lack of *mel* operon expression in *adpA* mutants of *S. coelicolor* ZD2, *S. lividans* ZD3, and *S. antibioticus* ZD4 compared with their progenitors, M145 (left), JT46 (middle), and IMRU3720 (right), respectively. (B) Dosage effect of *adpA*. The top half of each plate contained wild-type *S. antibioticus* IMRU3720, *S. lividans* JT46 and ZX64 carrying pIJ702, or *S. lividans* ZX64 and ZX66 carrying pIJ702, while the bottom half of each plate contained strains corresponding to the strains in the top half but with an additionally integrated copy of *adpA*₁ (carried on pJTU1457) except that the two patches for the same strain (ZX64/pIJ702::pJTU1457) are shown in the bottom half of the left plate. For the strains at the bottom there was both increased production of black pigment and enhanced sporulation compared with the strains at the top.

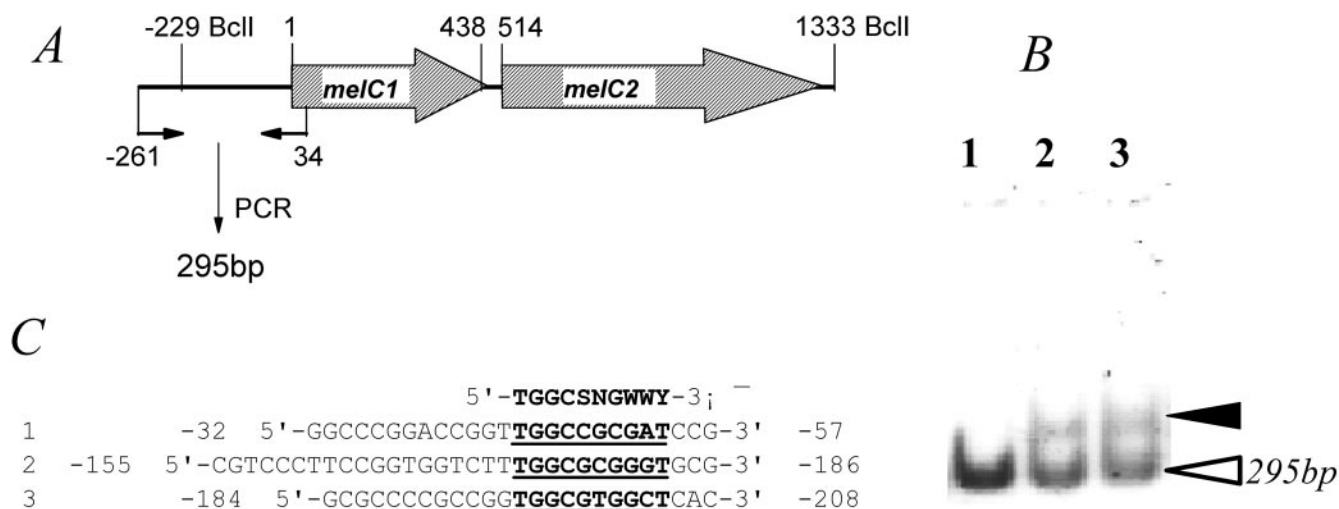


FIG. 5. (A and B) Gel mobility shift assay (B) for detection of the binding of AdpA₁ to a γ -³²P-labeled 295-bp DNA fragment (A) covering the promoter region of the *melC* operon. The solid arrowhead in panel B indicates the position when 0.01 μ g (lane 2) or 0.02 μ g (lane 3) of total proteins isolated from *E. coli* BL21(DE3) carrying pJTU1464 was added to the radioactively labeled probe fragment (open arrowhead). Addition of 0.02 μ g of total proteins isolated from *E. coli* BL21(DE3) carrying pET15b to the same probe was used as a negative control (lane 1). (C) Alignment of the three regions upstream of the *melC* operon (A) with the consensus AdpA-binding sequence (51) shown at the top (5'-TGGCSNGWWY-3', where S is G or C, W is A or T, Y is T or C, and N is any nucleotide).

DISCUSSION

The tyrosinase (*mel*) gene, which is responsible for melanin synthesis, has been sequenced from at least nine *Streptomyces* strains (*S. coelicolor*, *S. avermitilis*, *S. lincolnensis*, *S. galbus*, *S. castaneoglobisporus*, *S. tanashiensis*, *S. griseus*, *S. venezuelae*, *S. antibioticus*, and *S. glaucescens*). Both the gene sequences and the protein sequences of all of these organisms are similar, with similar sizes and with the genes organized as a polycistronic operon (*melC*) consisting of two separate ORFs (*melC1* and *melC2*), but little was known about the regulation of tyrosinase production at the molecular level in *Streptomyces*.

The initial isolation of many melanin-negative mutants of *S. griseus* revealed a close association among melanogenesis, the ability to produce streptomycin and A-factor, and the ability to form aerial mycelia (11). A-factor (2-isocaprolyl-3R-hydroxymethyl- γ -butyrolactone) (24) acts as a chemical signaling molecule that triggers morphological differentiation and secondary metabolism (6) at an extremely low concentration. A-factor exerts its signaling effects by binding to a cytoplasmic receptor protein (ArpA), which binds to the promoter region of a transcriptional regulator (AdpA_g), in order to dissociate it from the DNA and permit transcription of free AdpA_g to activate a number of genes required for both morphological differentiation and secondary metabolism (34). Several direct targets activated by AdpA have been identified, and these targets include *strR*, a pathway-specific transcriptional activator for streptomycin biosynthetic pathway genes in *S. griseus* (34); *sgmA*, a gene encoding a metalloendopeptidase that appears to affect the rate of development in *S. griseus* (22); *adsA* (*bldN* in *S. coelicolor* [4]), a gene encoding an RNA polymerase extracytoplasmic-function sigma factor needed for normal mycelial development in *S. griseus* (48); *ssgA*, which encodes a small acidic protein important for aerial hyphal septation in *S. coelicolor* (45) and *S. griseus* (21, 49); and *amfR*, a gene encoding a transcriptional activator of the *amf* operon needed for aerial mycelium formation in *S. griseus* (50). A direct link among melanogenesis, its absolutely required activation by the AdpA homolog, and its clear dosage effect either on melanin production or sporulation in a number of *Streptomyces* strains is obviously direct evidence that AdpA is a key and widespread transcription factor involved in multiple biological processes. A brief look at the promoter sequences upstream of the *melC* operon, whose DNA was used for the gel shift assay (Fig. 5), immediately revealed three regions with variable sequence homology to the identified consensus AdpA-binding sequence (5'-TGCSNGWWY-3') (51) (Fig. 5), which might be the specific target(s). The exact binding site, however, has not been determined by DNase I footprinting analysis.

S. coelicolor and *S. griseus* represent quite distant poles of *Streptomyces* phylogeny in terms of the 16S rRNA sequence, and their last common ancestor probably existed about 200 million years ago (10). However, homologs of the *S. griseus* *adsA*, *sgmA*, *amfR*, and *ssgA* genes and more than eight *adpA*-like genes are also found in the genome of *S. coelicolor*, and they may be involved in a number of independent or different aspects of the morphological and physiological development processes. The *adpA_c* gene, corresponding to *adpA_g* in *S. griseus* (41), was also found to have a rare TTA codon, like *adpA_l*. In *S. coelicolor*, mutation of *bldA*, which encodes the tRNA for

the rare leucine codon UUA, causes pleiotropic deficiencies in both the development of reproductive aerial hyphae and the production of antibiotics on most media (27, 33). As *adpA_l* also has a rare TTA codon like that found in *adpA_c* and *adpA_g*, it was not surprising to find that the *mel* gene was not expressed in a *bldA* mutant and hence was likely to be controlled at the translational level via *bldA* tRNA.

Comparative analysis of a single mutation resulted in identification of a critical nucleotide (A at position 214), whose change (to C) led to a transversion in the amino acid sequence (from Thr to Pro at position 27). Conceivably, this amino acid must be at the active site of the AdpA protein to activate the transcription of the *melC* operon and to affect sporulation, but whether it has a similar role in activating other biological process, such as antibiotic production, still needs to be examined closely.

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REFERENCES

- Altschul, S. F., and E. V. Koonin. 1998. Iterated profile searches with PSI-BLAST—a tool for discovery in protein databases. *Trends Biochem. Sci.* **23**:444–447.
- Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**:3389–3402.
- Bernan, V., D. Filpula, W. Herber, M. Bibb, and E. Katz. 1985. The nucleotide sequence of the tyrosinase gene from *Streptomyces antibioticus* and characterization of the gene product. *Gene* **37**:101–110.
- Bibb, M. J., V. Molle, and M. J. Buttner. 2000. σ^{bldN} , an extracytoplasmic function RNA polymerase sigma factor required for aerial mycelium formation in *Streptomyces coelicolor* A3(2). *J. Bacteriol.* **182**:4606–4616.
- Bierman, M., R. Logan, K. O'Brien, E. T. Seno, R. N. Rao, and B. E. Schoner. 1992. Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. *Gene* **116**:43–49.
- Chater, K. F., and S. Horinouchi. 2003. Signalling early developmental events in two highly diverged *Streptomyces* species. *Mol. Microbiol.* **48**:9–15.
- Chen, L. Y., M. Y. Chen, W. M. Leu, T. Y. Tsai, and Y. H. Lee. 1993. Mutational study of *Streptomyces* tyrosinase trans-activator MelC1. MelC1 is likely a chaperone for apotyrosinase. *J. Biol. Chem.* **268**:18710–18716.
- Chen, L. Y., W. M. Leu, K. T. Wang, and Y. H. Lee. 1992. Copper transfer and activation of the *Streptomyces* apotyrosinase are mediated through a complex formation between apotyrosinase and its trans-activator MelC1. *J. Biol. Chem.* **267**:20100–20107.
- della-Cioppa, G., S. J. Garger, G. G. Sverlow, T. H. Turpen, and L. K. Grill. 1990. Melanin production in *Escherichia coli* from a cloned tyrosinase gene. *Bio/Technology* **8**:634–638.
- Embley, T. M., and E. Stackebrandt. 1994. The molecular phylogeny and systematics of the actinomycetes. *Annu. Rev. Microbiol.* **48**:257–289.
- Endo, K., K. Kamo, K. Hosono, T. Beppu, and K. Ueda. 2001. Characterization of mutants defective in melanogenesis and a gene for tyrosinase of *Streptomyces griseus*. *J. Antibiot. (Tokyo)* **54**:789–796.
- Friedman, D. I., and M. Gottesman. 1983. Lytic mode of lambda development, p. 21–51. In R. W. Hendrix, J. W. Roberts, F. W. Stahl, and R. A. Weisberg (ed.), *Lambda II*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Gottesman, S., E. Roche, Y. Zhou, and R. T. Sauer. 1998. The ClpXP and ClpAP proteases degrade proteins with carboxy-terminal peptide tails added by the SsrA-tagging system. *Genes Dev.* **12**:1338–1347.
- Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* **41**:95–98.
- Hawkins, F. K., and A. W. Johnston. 1988. Transcription of a *Rhizobium leguminosarum* biovar *phaseoli* gene needed for melanin synthesis is activated by *nifA* of *Rhizobium* and *Klebsiella pneumoniae*. *Mol. Microbiol.* **2**:331–337.
- Hintermann, G., M. Zatchej, and R. Hutter. 1985. Cloning and expression of

- the genetically unstable tyrosinase structural gene from *Streptomyces glaucescens*. *Mol. Gen. Genet.* **200**:422–432.
17. Hobbs, G., C. M. Frazer, D. C. J. Gardner, J. A. Cullum, and S. G. Oliver. 1989. Dispersed growth of *Streptomyces* in liquid culture. *Appl. Microbiol. Biotechnol.* **31**:272–277.
 18. Hopwood, D. A. 1967. Genetic analysis and genome structure in *Streptomyces coelicolor*. *Bacteriol. Rev.* **31**:373–403.
 19. Horvath, M. M., and N. V. Grishin. 2001. The C-terminal domain of HPII catalase is a member of the type I glutamine amidotransferase superfamily. *Proteins* **42**:230–236.
 20. Ishikawa, J., and K. Hotta. 1999. FramePlot: a new implementation of the frame analysis for predicting protein-coding regions in bacterial DNA with a high G+C content. *FEMS Microbiol. Lett.* **174**:251–253.
 21. Jiang, H., and K. E. Kendrick. 2000. Characterization of *ssfR* and *ssgA*, two genes involved in sporulation of *Streptomyces griseus*. *J. Bacteriol.* **182**:5521–5529.
 22. Kato, J. Y., A. Suzuki, H. Yamazaki, Y. Ohnishi, and S. Horinouchi. 2002. Control by A-factor of a metalloendopeptidase gene involved in aerial mycelium formation in *Streptomyces griseus*. *J. Bacteriol.* **184**:6016–6025.
 23. Katz, E., C. J. Thompson, and D. A. Hopwood. 1983. Cloning and expression of the tyrosinase gene from *Streptomyces antibioticus* in *Streptomyces lividans*. *J. Gen. Microbiol.* **129**:2703–2714.
 24. Khokhlov, A. S., I. I. Tovarova, L. N. Borisova, S. A. Pliner, L. N. Shevchenko, E. Kornitskaia, N. S. Ivkina, and I. A. Rapoport. 1967. The A-factor, responsible for streptomycin biosynthesis by mutant strains of *Actinomyces streptomycini*. *Dokl. Akad. Nauk SSSR* **177**:232–235.
 25. Kieser, T., D. A. Hopwood, H. M. Wright, and C. J. Thompson. 1982. pIJ101, a multi-copy broad host-range *Streptomyces* plasmid: functional analysis and development of DNA cloning vectors. *Mol. Gen. Genet.* **185**:223–228.
 26. Kieser, T., M. J. Bibb, M. J. Buttner, K. F. Chater, and D. A. Hopwood. 2000. Practical *Streptomyces* genetics. The John Innes Foundation, Norwich, United Kingdom.
 27. Lawlor, E. J., H. A. Baylis, and K. F. Chater. 1987. Pleiotropic morphological and antibiotic deficiencies result from mutations in a gene encoding a tRNA-like product in *Streptomyces coelicolor* A3(2). *Genes Dev.* **1**:1305–1310.
 28. Lee, Y. H., B. F. Chen, S. Y. Wu, W. M. Leu, J. J. Lin, C. W. Chen, and S. C. Lo. 1988. A trans-acting gene is required for the phenotypic expression of a tyrosinase gene in *Streptomyces*. *Gene* **65**:71–81.
 29. Lerch, K. 1981. Metal ions in biological systems, vol. 13. Marcel Dekker Inc., New York, N.Y.
 30. Leu, W. M., L. Y. Chen, L. L. Liaw, and Y. H. Lee. 1992. Secretion of the *Streptomyces* tyrosinase is mediated through its trans-activator protein, MelC1. *J. Biol. Chem.* **267**:20108–20113.
 31. Lomovskaya, N. D., N. M. Mkrtumian, N. L. Gostimskaya, and V. N. Danilenko. 1972. Characterization of temperate actinophage ϕ C31 isolated from *Streptomyces coelicolor* A3(2). *J. Virol.* **9**:258–262.
 32. Mason, H. S. 1965. Oxidases. *Annu. Rev. Biochem.* **34**:595–634.
 33. Merrick, M. J. 1976. A morphological and genetic mapping study of bald colony mutants of *Streptomyces coelicolor*. *J. Gen. Microbiol.* **96**:299–315.
 34. Ohnishi, Y., S. Kameyama, H. Onaka, and S. Horinouchi. 1999. The A-factor regulatory cascade leading to streptomycin biosynthesis in *Streptomyces griseus*: identification of a target gene of the A-factor receptor. *Mol. Microbiol.* **34**:102–111.
 35. Paget, M. S., L. Chamberlin, A. Atrih, S. J. Foster, and M. J. Buttner. 1999. Evidence that the extracytoplasmic function sigma factor σ^{EF} is required for normal cell wall structure in *Streptomyces coelicolor* A3(2). *J. Bacteriol.* **181**:204–211.
 36. Paget, M. S., G. Hintermann, and C. P. Smith. 1994. Construction and application of streptomycete promoter probe vectors which employ the *Streptomyces glaucescens* tyrosinase-encoding gene as reporter. *Gene* **146**:105–110.
 37. Pearson, W. R. 1990. Rapid and sensitive sequence comparison with FASTP and FASTA. *Methods Enzymol.* **183**:63–98.
 38. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 39. Schaffer, A. A., L. Aravind, T. L. Madden, S. Shavirin, J. L. Spouge, Y. I. Wolf, E. V. Koonin, and S. F. Altschul. 2001. Improving the accuracy of PSI-BLAST protein database searches with composition-based statistics and other refinements. *Nucleic Acids Res.* **29**:2994–3005.
 40. Sun, Y., X. Zhou, J. Liu, K. Bao, G. Zhang, G. Tu, T. Kieser, and Z. Deng. 2002. *Streptomyces nanchangensis*, a producer of the insecticidal polyether antibiotic nanchangmycin and the antiparasitic macrolide meilingmycin, contains multiple polyketide gene clusters. *Microbiology* **148**:361–371.
 41. Takano, E., M. Tao, F. Long, M. J. Bibb, L. Wang, W. Li, M. J. Buttner, Z. X. Deng, and K. F. Chater. 2003. A rare leucine codon in *adpA* is implicated in the morphological defect of *bldA* mutants of *Streptomyces coelicolor*. *Mol. Microbiol.* **50**:475–486.
 42. Thompson, C. J., J. M. Ward, and D. A. Hopwood. 1980. DNA cloning in *Streptomyces*: resistance genes from antibiotic-producing species. *Nature* **286**:525–527.
 43. Tsai, J. F., and C. W. Chen. 1987. Isolation and characterization of *Streptomyces lividans* mutants deficient in intraplasmid recombination. *Mol. Gen. Genet.* **208**:211–218.
 44. Tseng, H. C., and C. W. Chen. 1991. A cloned *ompR*-like gene of *Streptomyces lividans* 66 suppresses defective *melC1*, a putative copper-transfer gene. *Mol. Microbiol.* **5**:1187–1196.
 45. van Wezel, G. P., J. van der Meulen, S. Kawamoto, R. G. Luiten, H. K. Koerten, and B. Kraal. 2000. *ssgA* is essential for sporulation of *Streptomyces coelicolor* A3(2) and affects hyphal development by stimulating septum formation. *J. Bacteriol.* **182**:5653–5662.
 46. Vujaklija, D., S. Horinouchi, and T. Beppu. 1993. Detection of an A-factor-responsive protein that binds to the upstream activation sequence of *strR*, a regulatory gene for streptomycin biosynthesis in *Streptomyces griseus*. *J. Bacteriol.* **175**:2652–2661.
 47. Willey, J., J. Schwedock, and R. Losick. 1993. Multiple extracellular signals govern the production of a morphogenetic protein involved in aerial mycelium formation by *Streptomyces coelicolor*. *Genes Dev.* **7**:895–903.
 48. Yamazaki, H., Y. Ohnishi, and S. Horinouchi. 2000. An A-factor-dependent extracytoplasmic function sigma factor ($\sigma^{A_{dsA}}$) that is essential for morphological development in *Streptomyces griseus*. *J. Bacteriol.* **182**:4596–4605.
 49. Yamazaki, H., Y. Ohnishi, and S. Horinouchi. 2003. Transcriptional switch on of *ssgA* by A-factor, which is essential for spore septum formation in *Streptomyces griseus*. *J. Bacteriol.* **185**:1273–1283.
 50. Yamazaki, H., Y. Takano, Y. Ohnishi, and S. Horinouchi. 2003. *anfR*, an essential gene for aerial mycelium formation, is a member of the AdpA regulon in the A-factor regulatory cascade in *Streptomyces griseus*. *Mol. Microbiol.* **50**:1173–1187.
 51. Yamazaki, H., A. Tomono, Y. Ohnishi, and S. Horinouchi. 2004. DNA-binding specificity of AdpA, a transcriptional activator in the A-factor regulatory cascade in *Streptomyces griseus*. *Mol. Microbiol.* **53**:555–572.