Enhanced Expression of S-Adenosylmethionine Synthetase Causes Overproduction of Actinorhodin in Streptomyces coelicolor A3(2)†

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We found that a 46-kDa protein is highly expressed in an actinorhodin-overproducing *Streptomyces coelicolor* A3(2) mutant (KO-179), which exhibited a low-level resistance to streptomycin. The protein was identified as *S*-adenosylmethionine (SAM) synthetase, which is a product of the *metK* gene. Enzyme assay revealed that SAM synthetase activity in strain KO-179 was 5- to 10-fold higher than in wild-type cells. The elevation of SAM synthetase activity was found to be associated with an increase in the level of intracellular SAM. RNase protection assay revealed that the *metK* gene was transcribed from two distinct promoters (p1 and p2) and that enhanced expression of the MetK protein in the mutant strain KO-179 was attributed to elevated transcription from *metK*p2. Strikingly, the introduction of a high-copy-number plasmid containing the *metK* gene into wild-type cells resulted in a precocious hyperproduction of actinorhodin. Furthermore, the addition of SAM to the culture medium induced Act biosynthesis in wild-type cells. Overexpression of *metK* stimulated the expression of the pathway-specific regulatory gene *act*II-ORF4, as demonstrated by the RNase protection assay. The addition of SAM also caused hyperproduction of streptomycin in *Streptomyces griseus*. These findings implicate the significant involvement of intracellular SAM in initiating the onset of secondary metabolism in *Streptomyces*.

Streptomyces coelicolor A3(2) is the genetically best-characterized streptomycete (4, 34) and is frequently used as a model organism for studying the regulation of antibiotic production (5, 15). This strain produces at least four chemically distinct classes of antibiotics, two of which are pigmented: the blue polyketide actinorhodin (Act) and the red tripyrrole undecylprodigiosin (Red). This fact provides a feasible system for the study of pathway-specific and pleiotropic regulation of antibiotic synthesis.

We reported previously that introduction of certain *str* mutations, which is able to confer resistance to streptomycin, enhance Act production in *Streptomyces lividans* and *S. coelicolor* A3(2) (14, 37). Furthermore, the impaired ability to produce Act due to certain developmental mutations (*relA*, *relC*, and *brgA*) in *S. coelicolor* can be circumvented by introducing the *str* mutations (28, 30, 37). These *str* mutants are classified into two types. The type I mutants (*rpsL* mutants) have a mutation within the *rpsL* gene which encodes the ribosomal protein S12 and demonstrates a high-level of resistance (>100 μg/ml) to streptomycin. The type II mutants possess the wild-type *rpsL* gene and demonstrates a low level of resistance (5 to 10 μg/ml). Although a type II mutation in *S. coelicolor* KO-132 (*relA str-1*) had been genetically mapped on the genetic map (28), the actual mutation site (i.e., a mutated gene) remains unknown. However, the appearance of the type II mutants is frequent (ranging from 10^{-5} to 10^{-7}), and their ability to produce Act is noticeably higher than in the *rpsL* mutants.

Understanding the detailed mechanism that underline the altered cellular processes will lead to the elucidation of how enhanced production of Act occurs in the type II mutants. We report here the identification of a specific (46-kDa) protein, which is highly expressed in the *S. coelicolor* type II mutant KO-179. This protein is shown to be *S*-adenosylmethionine (SAM) synthetase (EC 2.5.1.6), a product of the *metK* gene. The possible involvement of the level of intracellular SAM in the regulation of Act production is also discussed, according to results from physiological and molecular level analysis.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains of *S. coelicolor* A3(2) used in the present study were 1147 (prototrophic wild-type strain) and its derivative KO-179 (*str-19*) (14). *Streptomyces griseus* IFO13189, a wild-type prototrophic strain (26), was used for the production of streptomycin. *E. coli* strains DH5α and GM2163 were used for routine DNA manipulation. Spores from these *S. coelicolor* strains were harvested from cultures grown on GYM (37) or SFM (19) medium. R4 (37), R4C (R4 supplemented with 0.5% Casamino Acids), and R5⁻ medium (17) were used for Act production. SMMS (19) was used for calcium-dependent antibiotic (CDA) production. When necessary, *S. coelicolor* cultures were grown on an agar medium covered with a cellophane sheet, after being inoculated with ca. 10^6 spores, and incubated at 30°C. Act, Red, and CDA were determined according to the method of Kieser et al. (19). Production of streptomycin was performed as described previously (26).

Isolation and manipulation of DNA and RNA. Plasmid isolation, restriction enzyme digestion, ligation, and transformation of *Escherichia coli* and *Streptomyces* were carried out by the methods of Sambrook et al. (36) and Kieser et al. (19), respectively. PCR was performed by using a TaKaRa LA *Taq* or TaKaRa Ex *Taq* enzyme with GC Buffer I (Takara, Tokyo, Japan). Unless stated otherwise, genomic DNA prepared from strain 1147 was used as a template for PCR. For RNA extraction, mycelium (ca. 50 to 100 mg of wet weight) from the plate culture (see above) was suspended in 1 ml of Isogen reagent (Nippon Gene, Toyama, Japan), and 0.2 g of zirconium beads (diameter, 0.5 mm) was added. The samples were treated at 2,000 rpm for 3 min in a Beads Homogenizer (Central Scientific Commerce, Tokyo, Japan), and then RNA was isolated according to the manufacturer's instructions.

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Plasmid construction. Plasmid pXEmetK, containing a *metK-xylE* transcriptional fusion element, was constructed as follows. The *metK* promoter region was generated by PCR with the primers PF1+*Bam*HI (5′-GGATCCAGCGATCG GTTGTCGGGGTG-3′) and PR-1+*Hin*dIII (5′-AAGCTTCGGCTCCGAGGA GAAC-3′). A 435-bp PCR product was cloned in pCR2.1 TA cloning vector (Invitrogen, Carlsbad, Calif.). After sequence confirmation, the *metK* promoter fragment was excised by *Bam*HI and *Hin*dIII digestion and cloned into the pXE4 promoter probe vector (18) to generate pXEmetK. After passage through *E. coli* GM2163 (Δ*dam* Δ*dcm*), the plasmid pXEmetK was introduced into *S. coelicolor* strains.

The high-copy-number plasmid pMetK1, containing the *S. coelicolor metK* gene, was constructed as follows. The cosmid clone SCL6 (34) was digested with *Sap*I, treated with Klenow enzyme to generate blunt ends, and digested with *Not*I, resulting in a 2.3-kb *metK*-containing fragment. This fragment was ligated with pBluescript II SK(+) treated with *Not*I and *Eco*RV, resulting in pBlueMetK2. The *metK* gene was excised from the pBluMetK2 by *Sac*I and *Hin*dIII digestion and cloned in pIJ487 (43) treated with the same enzymes to generate pMetK1.

Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE). Cells were harvested from the R4 liquid culture, resuspended in lysis solution (8 M urea, 4% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, 40 mM Tris base), and disrupted by sonication for 2 min. After centrifugation at 15,000 × *g* for 10 min at 4°C, the supernatants were used as protein extract samples. A 100-μg portion of the total proteins was subjected to first-dimension isoelectric focusing (IEF) electrophoresis. IEF electrophoresis was performed with an Immobiline dry strip (pH 4 to 7, 11 cm; Amersham Biosciences, Piscataway, N.J.) by using a Multiphor II Electrophoresis Unit (Amersham Biosciences). An ExcelGel sodium dodecyl sulfate (SDS) Gradient 12-14 (Amersham Biosciences) was used for 2-D SDS-PAGE. Proteins were detected by staining with Coomassie brilliant blue R-250.

Determination of intracellular SAM. Intracellular SAM levels were determined by reversed-phase high-pressure liquid chromatography by a method described previously (32). Cells were grown on R5⁻ agar covered with cellophane. At the times indicated (36, 48, 60, and 72 h after inoculation), the cellophane sheet with cells was pealed from the plate and immediately transferred to a petri dish containing 10 ml of 1 M formic acid, followed by standing at 4°C for 1 h. The formic acid was collected, filtered through a 0.45-μm-pore-size filter, and then lyophilized. The lyophilized samples were dissolved in a small amount of water and analyzed with a Capcell-Pak C_{18} column (4.6 by 250 mm; Shiseido, Tokyo, Japan).

Enzyme assays. Cells harvested from plate culture were suspended in 0.2 M 2-(*N*-morpholino)ethanesulfonic acid buffer (pH 6.0), and sonicated. Crude cell extract was obtained by centrifugation at 15,000 × *g* for 10 min at 4°C and used for the enzyme assay. SAM synthetase was assayed by spectrophotometrically measuring pyrophosphate production by using the Sigma pyrophosphate reagent (21). XylE enzyme activity was assayed as described previously (19), except that cells were grown on R5⁻ agar.

Western blot analysis. A portion (15 μg) of total proteins was subjected to 15% (for ActII-ORF4 protein) or 12% (for ActI-ORF1 protein) polyacrylamide-sodium dodecyl sulfate gels. Western blot analyses were performed as described previously (16, 31, 46).

RNase protection assays. A *metK* riboprobe was generated as follows. The SCL6.34c-*metK* intergenic region was amplified by PCR with the primers *Eco*RI-metKp-F1 (5′-CTCT<u>GAATTC</u>CCATTTGCTCGAAGGAGCAG-3′) and *Hin*-dIII-metKp-R1 (5′-CTCT<u>AAGCTT</u>ACCAGACCGGTGGTGATCA-3′) to produce a 406-bp fragment containing *Eco*RI and *Hin*dIII sites (indicated by underlining). This fragment was digested with *Eco*RI and *Hin*dIII and inserted into pUC19. The resultant plasmid (pMetKP2) was sequenced and used for PCR amplification with the primers M13(-40) and *Hin*dIII-metKp-R1 to produce a 439-bp fragment. A T7 RNA polymerase promoter sequence was introduced into this fragment by using the Lig′nScribe no-cloning promoter addition kit (Ambion, Austin, Tex.), and in vitro transcription was carried out with a MAXIscript in vitro transcription kit (Ambion) and labeled with [α-³²P]UTP (Amersham Biosciences). The *metK* riboprobe (449 nucleotide [nt]) contained a 62-nt non-homologous sequence from the pUC19 multiple cloning site and T7 promoter sequence. A 257-bp fragment containing the *hrdB* promoter region was generated by PCR with the primers RPA-hrdB-F (5′-GATTGGGCGTAACGCTCT TG-3′) and RPA-hrdB-R (5′-CCATGACAGAGACGGACTCG-3′). A T7 promoter sequence was introduced to this 257-bp fragment by using the Lig′nScribe kit, and an in vitro transcription reaction was performed as described above. An *act*II-ORF4 riboprobe was generated from a 267-bp PCR fragment amplified with the primers RPA-actII-F (5′-AATCTGTTGAGTAGGCCTGTT-3′) and RPA-actII-R (5′-GTACACGTACGTCTGCAGC-3′) in a similar way.

RNA samples (20 μg each) were hybridized overnight at 42°C with labeled riboprobe (5 × 10⁴ cpm) by using the RPA III RNase protection assay kit (Ambion). After hybridization, samples were digested with an RNase mixture (RNase A and RNase T_1) according to the manufacturer's instructions. Digested samples were precipitated, redissolved in gel-loading buffer, and loaded on a 5% polyacrylamide–8 M urea gel. ³²P-labeled RNA molecular size markers were generated by in vitro transcription using a RNA Century Marker template sets (Ambion). Radioactivity on gels were quantitated with a STORM 860 image analyzer (Molecular Dynamics, Sunnyvale, Calif.).

Primer extension analysis. Primer extension analysis was done as described in Sambrook et al. (36). The primer (5′-TCACGGACTCCGAGGTGAACAG-3′) was labeled with [γ-³²P]ATP by using T4 polynucleotide kinase. Total RNA (40 μg) was hybridized with 5′ end-labeled primer (ca. 10⁵ cpm) and used for the primer extension reaction. The extension products were analyzed in a sequencing gel (6% polyacrylamide–8 M urea) with sequencing ladders generated from the same primer.

RESULTS

2-D PAGE analysis of total proteins. Several Act-overproducing mutants selected for low-level resistance to streptomycin have been isolated and characterized in our laboratory (14, 28, 37). These mutants, unlike the mutants selected for high-level resistance, have no mutation within the *rpsL* gene that encodes the ribosomal protein S12, even though resistance to streptomycin has been known to result frequently from mutations in the *rpsL* gene (14, 37). We tentatively designated the mutation(s) in these low-level *str* mutants as "ND." KO-179 is a representative strain from the ND mutant group, which were derived from strain 1147, a prototrophic wild-type strain of *S. coelicolor* A3(2). KO-179 produces a greater amount of Act than the wild-type strain in both solid and liquid cultures but shows reduced sporulation (14).

We first compared protein expression profiles from KO-179 and the wild-type strain 1147 by using 2-D PAGE. Strains were grown to early (6 h), middle (20 h), or late (32 h) growth phase in liquid R4 medium, and the total cellular proteins extracted were analyzed by 2-D PAGE in a pH range of between 4 and 7. We found that a 46-kDa protein exists in abundance in KO-179 but not in the wild-type strain, as indicated by the arrows in Fig. 1. The pI value of this protein was estimated to be 4.5 to 5.0. We also found that ActVI-ORF3, a putative protein for Act biosynthesis (4), increased five- to sevenfold as determined by 1-D PAGE (data not shown). The expression of this 46-kDa protein appeared to be temporal since a burst of expression was detected only in the late growth phase (20 to 32 h) but not in the early growth phase (6 h). Thus, we assumed that the 46-kDa protein may play a role during a late-growth-phase-specific cell event such as antibiotic production.

Identification of the 46-kDa protein. For N-terminal sequencing, the 46-kDa protein spot was excised from the 2-D gel and analyzed on a protein sequencer. The N-terminal sequence was found to be XRRLFTXESVTE, which is highly homologous with the *metK* gene product, SAM synthetase (accession no. CAB76898). The *S. coelicolor metK* gene codes for a 404-amino-acid protein with a predicted molecular mass of 43 kDa. The predicted isoelectric point for the MetK protein is 4.7. These values all agree well with the experimental data for our 46-kDa protein.

SAM synthetase activity and the level of intracellular SAM in KO-179. To ascertain whether the level of SAM synthetase increased in the mutant strain KO-179, we measured the enzyme activity. Parent (strain 1147) and mutant (strain KO-179)
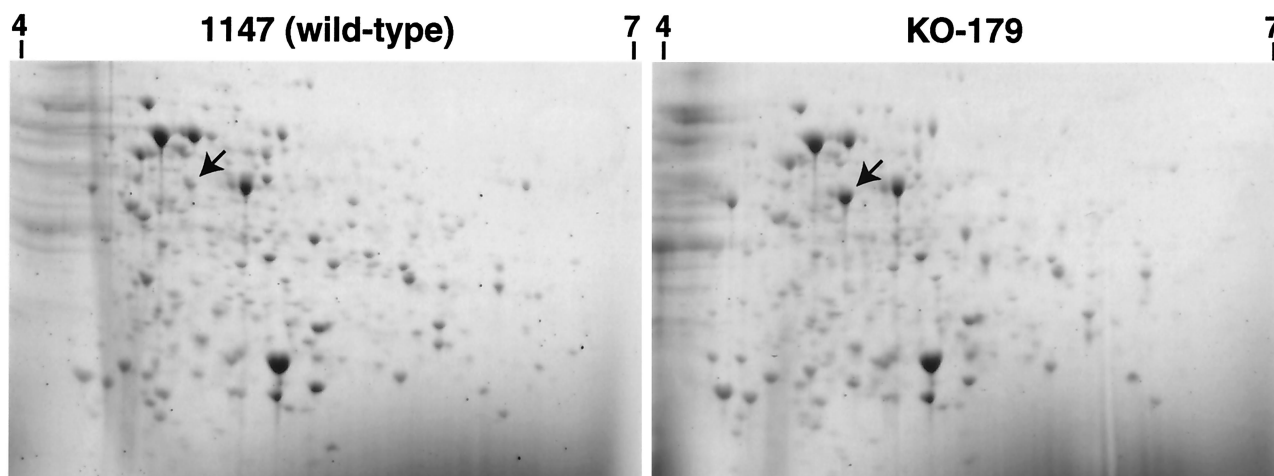
FIG. 1. Analysis of total cellular proteins by 2-D PAGE. Cells were grown in liquid R4 medium and harvested at 30 h (the time of entry into the stationary phase). Total proteins from *S. coelicolor* 1147 (wild-type) and the mutant KO-179 were analyzed by 2-D PAGE. MetK protein is indicated by the arrows. The markers, 4 and 7, on the figures indicate the isoelectric pH points.

strains were grown in R5⁻ agar medium covered with cellophane, and mycelia were harvested at various growth phases, followed by measurement of SAM synthetase activity. It is evident from Table 1 that SAM synthetase activity in KO-179 increased remarkably throughout the whole-cell cycle, ranging from 5- to 10-fold, thus reflecting the observed overexpression of the MetK protein.

We next asked whether or not the intracellular SAM level was actually altered upon the overexpression of SAM synthetase. Strains KO-179 and 1147 were grown on R5⁻ agar medium as described above, and the intracellular SAM level was determined (see Materials and Methods). As expected, the level of intracellular SAM in KO-179 was found to be markedly elevated relative to the wild-type strain (Table 1). This was especially pronounced in the late growth phase (36 to 48 h), at which time Act production commences. These results led us to the hypothesis that the increased level of SAM may be responsible for the observed activation of Act biosynthesis.

Transcription of the *metK* gene is elevated in KO-179. To address the question whether overproduction of SAM synthetase in KO-179 is exerted at the transcription level, we monitored the expression of *xylE* (which encodes catechol di-

oxigenase) under the control of the *metK* promoter. Wild-type and KO-179 cells were transformed with pXEmetK, a *xylE* expression plasmid containing a 425-bp putative *metK* promoter fragment. In the plate assay, KO-179 cells containing pXEmetK was shown to produce a bright yellow color upon exposure to catechol when grown on R5⁻ agar medium, whereas no catechol dioxigenase activity was detected in the wild-type strain containing the same plasmid. This finding indicates that the 425-bp *metK* promoter is functional and that transcription of the *metK* gene was significantly accelerated in the mutant strain KO-179. Quantitative assay revealed only slight catechol dioxigenase activity in the parental strain 1147 throughout the course of the experiment (Fig. 2). In contrast, significant catechol dioxigenase activity was detected when expression of the *metk-xylE* element was assessed in the ND mutant KO-179. The highest activity was found in the early-

TABLE 1. SAM synthetase activity and the level of intracellular SAM in *S. coelicolor* KO-179 and the parental strain 1147[a]

Time (h)	SAM synthetase activity (mU/mg of protein)[b]		SAM level (pmol/mg [wet wt])	
	1147	KO-179	1147	KO-179
24	0.91	4.01	ND	ND
36	0.97	10.1	50.7	198.9
48	2.27	15.4	45.5	132.4
60	1.05	10.4	117.7	77.5
72	1.46	6.14	62.0	48.5

[a] Cells were grown on R5⁻ agar covered with cellophane. Samples were taken at the indicated times, and the SAM synthetase activity and intracellular SAM content were determined as described in Materials and Methods. ND, not determined due to an insufficient amount of cells.

[b] One unit of activity is defined as the amount of enzyme that produced an change in optical density at 340 nm of 12.44/min.
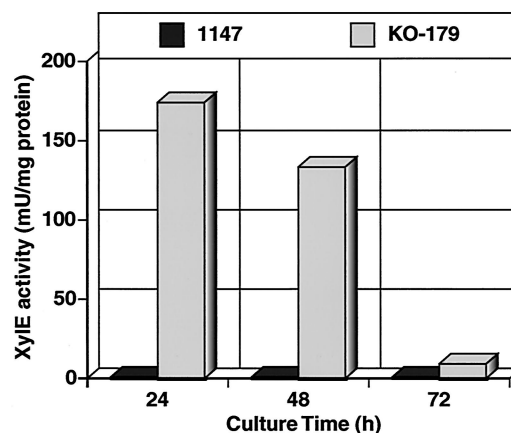

FIG. 2. Expression of *metK-xylE* fusion element. The graph shows results for the quantitative catechol dioxigenase assays for expression of the *metK-xylE* fusion element in strains 1147 and KO-179. Cells were grown in R5⁻ agar medium.
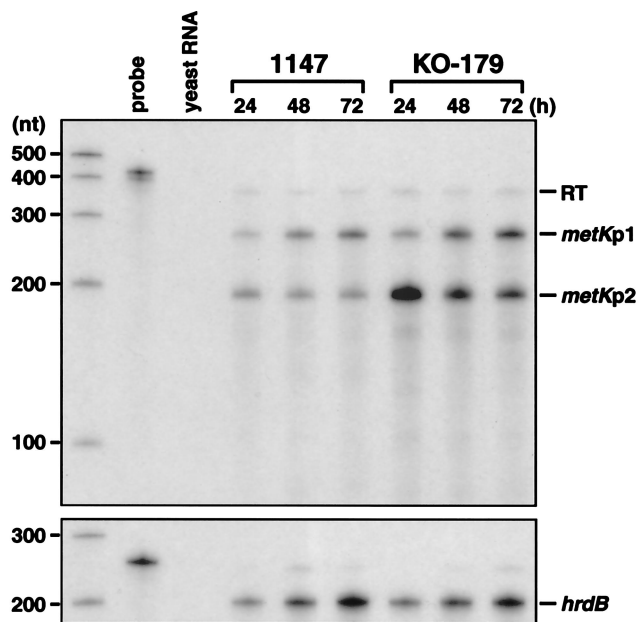
FIG. 3. Expression of *metK* in strains 1147 and KO-179. Transcription of *metK* during growth on R5⁻ agar medium was analyzed by RNase protection assay. Transcripts originating from two distinct *metK* promoters (*metK*p1 and *metK*p2) are indicated, together with readthrough transcription (RT) from the upstream gene (SCL6.34c). *hrdB* mRNA was determined as an internal control.

growth-phase cells, and the activity decreased gradually with time.

Expression of the *metK* gene was further examined by RNase protection assay with total RNA isolated from the indicated cells. A 449-nt riboprobe used in the RNase protection assay covers the SCL6.34c-*metK* intergenic region, including 62 nt of the nonhomologous tail that is derived from vector and

T7 promoter sequences (see Materials and Methods). As shown in Fig. 3, three protected fragments (350, 260, and 195 nt) were detected from the RNA isolated from 1147 and KO-179. In our electrophoresis system, RNA fragments derived from *Streptomyces* sequences migrate faster than the RNA marker used in the experiment, and the relative mobilities differ by ca. 15% (data not shown). Therefore, actual sizes of the protected fragment should be about 390, 300, and 225 nt, respectively. The largest protected fragment that corresponds to full-length protection over the homologous region of the riboprobe (387 nt) apparently indicates readthrough transcription from the SCL6.34c gene, whereas the other two protected fragments are considered to represent transcription from their own promoters. Precise start sites of transcription were determined by primer extension analysis (Fig. 4A). The start sites identified were 147 and 75 nt upstream from the GTG translation start codon (for *metK*p1 and *metK*p2, respectively), which corresponds to the positions predicted from the result of RNase protection assay (Fig. 4B). Thus, the *S. coelicolor metK* gene is transcribed from two distinct promoters, in addition to transcriptional readthrough from the upstream gene SCL6.34c.

The level of readthrough transcription was similar between KO-179 and the wild-type strain and was constant throughout the time course (Fig. 3). Transcription from *metK*p1 increased with time, and the expression level for KO-179 was somewhat higher than 1147. Interestingly, the amount of transcript generated from *metK*p2 was shown to be much greater in strain KO-179. The level of *metK*p2 transcript in KO-179 was about eightfold higher than strain 1147 in the early growth phase (24 h) and then decreased in the late growth phase. Transcription from *metK*p2 in strain 1147 was weak and constant. The observed expression pattern for *metK*p2 agrees well with the results of *metK*-*xylE* fusion assays (see above). These results suggest that enhanced expression of MetK protein in KO-179 is attributed to the elevated transcription from *metK*p2.

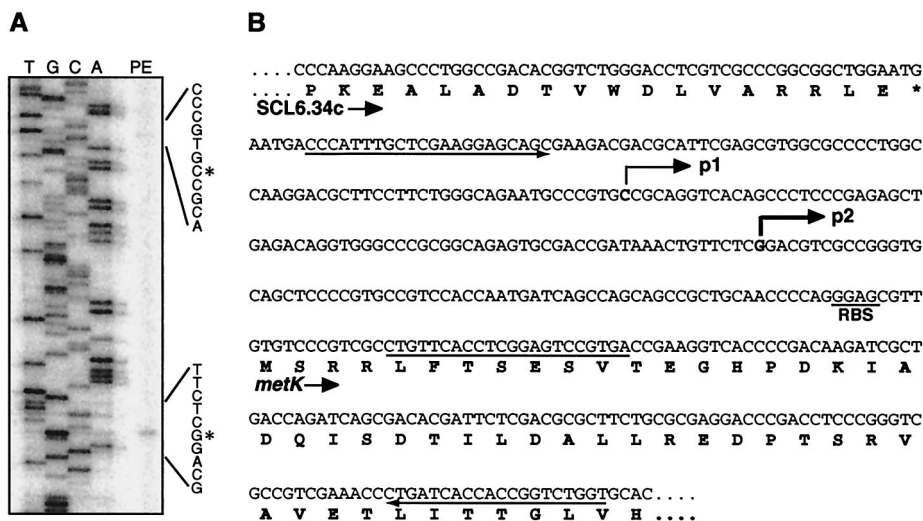It was possible from the results described above that the

FIG. 4. Transcription start points for *metK*. (A) Primer extension analysis of the transcription start points for *metK*p1 and *metK*p2. The start points assigned are indicated by asterisks. (B) Nucleotide sequence for the *metK* promoter region. Bent arrows indicate the transcription start points. The position at which the oligonucleotide used for the primer extension analysis annealed is underlined. The sequences from which primers were designed to generate the riboprobe for the RNase protection assay are denoted by the underline arrows.
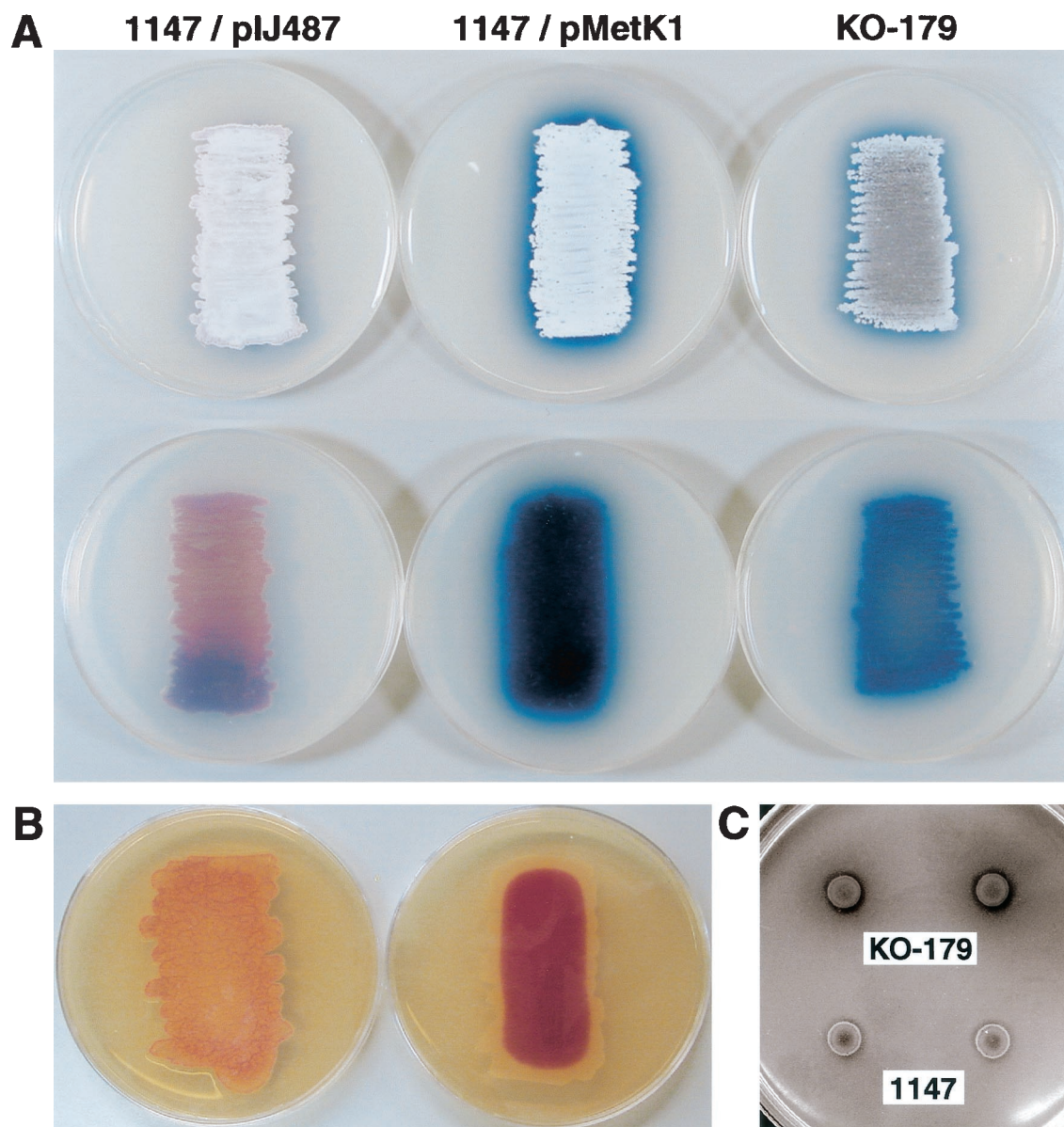
FIG. 5. (A) Effect of the introduction of a multicopy *metK* plasmid on the production of Act in wild-type strain 1147. Strains were inoculated on R4C agar medium, followed by incubation for 4 days at 30°C. 1147/pIJ487 represents the vector control. The upper panel indicates the top of the plate, whereas the lower panel represents the reverse side of the plate. (B) Red production in strains 1147 and KO-179. Strains were inoculated on GYM agar and grown at 30°C for 2 days (for KO-179) or 3 days (for 1147). The reverse side of plates is shown. (C) CDA production in strains 1147 and KO-179. Portions (5 μl) of spore suspensions (ca. 10^5 spores) were spotted onto SMMS plates and incubated at 30°C for 48 h. CDA production was detected as described previously (19).

mutant strain KO-179 may harbor a mutation in the promoter region of *metK* gene. However, sequencing analysis of the *metK* promoter region (and also *metK* open reading frame [ORF] region) could not detect any differences between KO-179 and the wild-type strain 1147 (data not shown).

Overexpression of the *metK* gene or addition of SAM causes overproduction of Act in wild-type cells. Because the MetK protein was identified as the protein that was highly expressed in the Act-overproducing mutant KO-179, MetK may be involved in this phenotype. To assess this possibility, we introduced a high-copy-number plasmid containing the *metK* gene

(pMetK1) into the wild-type strain 1147. Strikingly, introduction of pMetK1 into strain 1147 resulted in a precocious hyperproduction of Act, as determined in both solid culture (Fig. 5A) and liquid culture (data not shown). SAM synthetase activity from srain 1147/pMetK1 was twofold higher than cells carrying the vector plasmid pIJ487. Introduction of the *metK* promoter region alone into strain 1147 by using a multicopy plasmid did not influence Act production. Furthermore, introduction of pMeK1 into *S. lividans* TK21, a close relative of *S. coelicolor* A3(2), did induce Act production (data not shown). Strain 1147/pMetK1 (and also mutant strain KO-179) grew
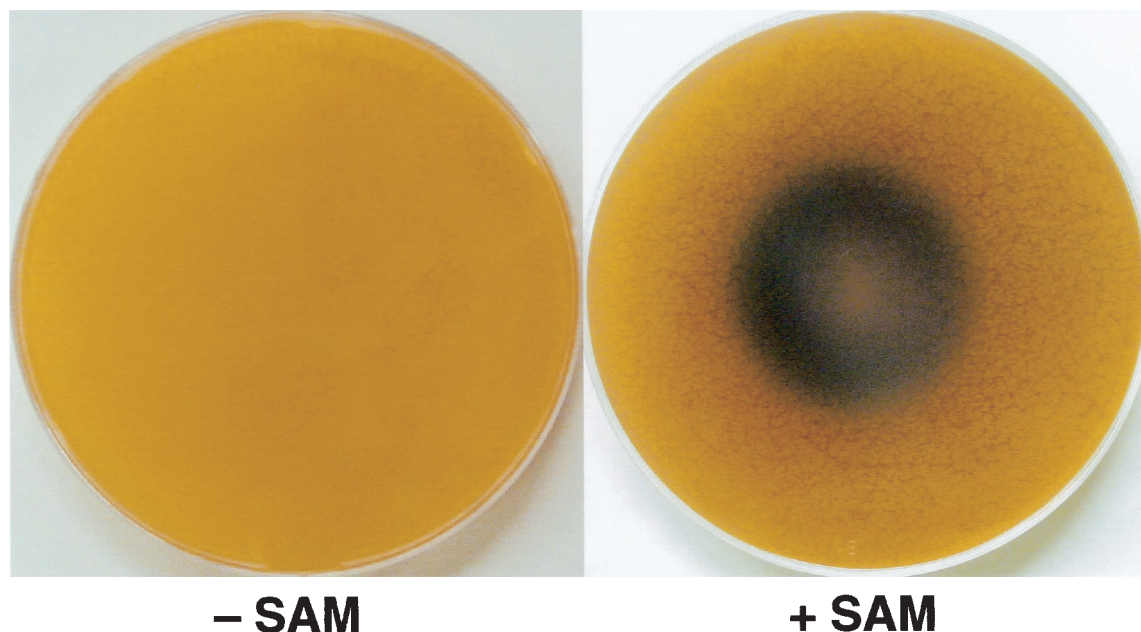
FIG. 6. Effect of exogenously added SAM. Strain 1147 spores were spread on GYM agar, and 3 mg of SAM (right plate) was placed in the center of the plate. The left plate shows a control plate without SAM. The plates were incubated at 30°C for 4 days. The reverse side of the plates is shown.

normally in minimal medium without demonstrating the need for methionine (data not shown). Also, there was no detectable difference in the level of resistance to streptomycin between strains 1147/pMetK1 and 1147/pIJ487 (MIC of streptomycin of 0.8 μg/ml for these strains but MIC of 5 μg/ml for strain KO-179), implying that the streptomycin-resistant phenotype in the ND mutant KO-179 did not result from the increase in intracellular SAM level.

Next, we examined the effect of exogenously added SAM on Act production. If *S. coelicolor* could take up SAM, then SAM addition could mimic the effect of MetK overexpression, resulting in the overproduction of Act. As expected, exogenous SAM actually did cause Act overproduction in the wild-type strain 1147 (Fig. 6). The concentrations for SAM that were effective for Act overproduction were 1 mM or higher, as determined by using plates with fixed concentrations of SAM (data not shown). To assess the possibility that the observed effects were exerted by a metabolic product(s) generated from SAM rather than a direct activity of SAM, we tested the effects of various SAM-related metabolites on Act production. None of the *S*-adenosylhomocysteine, homocysteine, cysteine, homoserine, methionine, ornithine, spermidine, putrescine, and adenosine (added at 1 mM) showed any effects on the ability to produce Act (data not shown). Thus, we concluded that the elevated intracellular SAM level per se caused by overexpression of MetK is responsible for the enhanced production of Act in mutant strain KO-179.

In considering the regulatory mechanism of SAM, it is important whether the effect of ND mutation in KO-179 is specific to Act or general to secondary metabolism in *S. coelicolor.* Therefore, we compared the ability of wild-type and mutant strains to produce Red and CDA, antibiotics produced by *S. coelicolor.* As shown in Fig. 5B, ND mutant KO-179 displayed

a precocious hyperproduction of Red, in addition to an increased productivity of CDA (Fig. 5C).

The efficacy of exogenous addition of SAM was also demonstrated in another streptomycete, *S. griseus*, a producer of streptomycin. The addition of 1 mM SAM into the culture medium at the time of inoculation resulted in a hyperproduction of streptomycin, as determined with both synthetic medium and nutrient medium (data not shown).

Effect of MetK overproduction on the expression of ActII-ORF4. Expression of the Act biosynthetic genes is controlled by the pathway-specific regulatory gene, *act*II-ORF4 (12). The ActII-ORF4 protein, a product of the *act*II-ORF4 gene, has been characterized as an OmpR-like DNA binding protein that positively regulates the transcription of the Act biosynthetic genes (3). To assess the possible role for the overproduction of SAM synthetase on the expression of ActII-ORF4, we grew cells (1147/pMetK1, 1147/pIJ487, and KO-179) on R4C agar covered with cellophane. Cells were harvested at various growth phases, and ActII-ORF4 levels were determined by Western blot analysis (Fig. 7A). Notably, *metK* overexpression was found to cause a significant increase in the expression of ActII-ORF4 compared to the control strain (1147/pIJ487). The mutant strain KO-179 also showed a marked increase in the expression of ActII-ORF4. The increase of ActI-ORF1 protein (a polyketide synthase subunit) was also more pronounced in both 1147/pMetK1 and KO-179 (Fig. 7A).

Finally, we conducted an RNase protection assay for the *act*II-ORF4 transcript by by using total RNA isolated from the cells used for Western blot analysis (see above). Compared to the control strain 1147/pIJ487, there was a marked increase in *act*II-ORF4 transcription in both 1147/pMetK1 and KO-179 (Fig. 7B). Thus, overexpression of *metK*, which in turn leads to
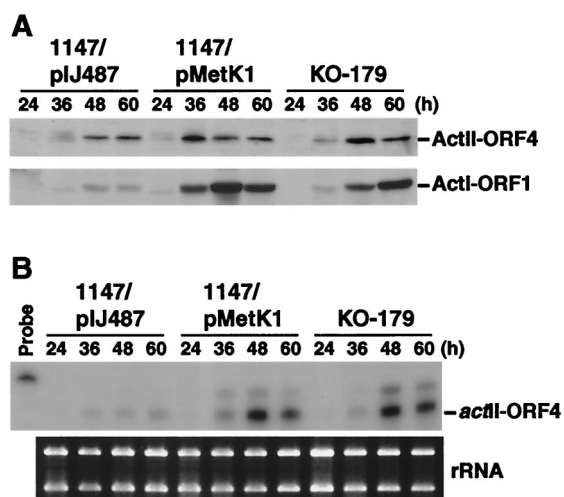
FIG. 7. Effect of the introduction of a multicopy *metK* plasmid on the expression of *act*II-ORF4. Cells were grown on R4C agar covered with cellophane and harvested at the indicated times. (A) Crude extracts were prepared and used for Western blot analysis to detect the ActII-ORF4 and ActI-ORF1 proteins. (B) RNA was isolated from the same culture and analyzed for the presence of *act*II-ORF4 mRNA by RNase protection assay. An ethidium bromide-stained gel containing 1 μg of RNA in each lane demonstrates the integrity of the RNA preparation.

an elevated intracellular SAM level, activates transcription of the pathway-specific regulatory gene, *act*II-ORF4.

DISCUSSION

The importance of SAM synthetase activity (and thus the level of SAM) in microbial development processes was first proposed 20 years ago by Ochi et al. (27, 29), particularly focusing on antibiotic production and sporulation in *Streptomyces griseoflavus* and *Bacillus subtilis*, respectively. The results described here establish the significance of SAM synthetase activity in initiating antibiotic production in *S. coelicolor* A3(2). Supporting this idea is the fact that streptomycin production by *S. griseus* is also enhanced by the addition of SAM to the culture medium. SAM is known to be the methyl donor for the methylation of cytosine and adenosine bases in DNA, rRNA, and tRNA, of various proteins, and of small molecules that are important in both lower and higher organisms (8, 25, 35, 42). Thus, the SAM synthetase is thought to be an essential enzyme for the viability of *E. coli* (44) and *B. subtilis* (47). Overexpression of the SAM synthetase gene in *E. coli* or *B. subtilis* is known to cause methionine auxotrophy (44, 47). An explanation for this auxotrophy is that overexpression of SAM synthetase depletes the intracellular methionine pool, either by consuming it to synthesize SAM or by repressing the methionine biosynthetic genes. In fact, SAM has been shown to be a corepressor in the mehionine regulation system in *E. coli* (13). Although *B. subtilis* showed a 6-fold increase in SAM synthetase activity that resulted in methionine auxotrophy, the *S. coelicolor* strain KO-179 produced 10-fold-greater SAM synthetase than did the wild-type strain (Table 1) without affecting growth as examined in minimal medium. Therefore, it is of

interest to determine whether SAM acts as a corepressor in the methionine regulation system in *Streptomyces*.

As shown by both the *metK-xylE* fusion analysis and the RNase protection assay, enhanced expression of MetK protein in KO-179 is apparently achieved at the transcriptional level by activation of a specified (*metK*p2) promoter. The start site for the *metK*p1 transcript is preceded by a −10 region (CAGAAT) that is similar to the consensus sequence (TAGYYT, Y = G or A) deduced from streptomycete promoters. This consensus sequence is probably recognized by the major RNA polymerase that contains sigma HrdB (38). However, the presence of the recognizable −35 sequence, which is often found in typical HrdB-dependent promoters, is lacking. The *metK*p2 also lacks obvious sequence similarity with other known streptomycete promoters in the −10 and −35 region. Although maximum SAM synthetase activity in KO-179 was detected at 48 h (Table 1), transcription from *metK*p2 reduced gradually after 24 h (Fig. 3). This implies that accumulation of the MetK protein during this period occurred. Since the *metK* promoter region in KO-179 has been shown not to have a mutation, it is conceivable that the enhanced transcription from *metK*p2 in KO-179 may be caused by an alteration in quality and/or quantity of a presumptive transcription regulator for *metK*, and this may be how the ND mutation functions to increase the production of Act.

Most interestingly, introduction of the multicopy *metK* plasmid into wild-type cells led to a remarkably abundant production of Act (Fig. 5A). Furthermore, the addition of SAM to the culture medium also induced Act biosynthesis (Fig. 6). Thus, enhanced Act production in mutant strain KO-179 is attributed, at least in part, to the overexpression of MetK protein, leading to an elevation of intracellular SAM level. In agreement with our conclusion, Kim et al. (20) reached a similar conclusion in working with *Streptomyces spectabilis*, a spectinomycin producer. These authors found that introduction of a multicopy plasmid containing the *S. spectabilis metK* gene into *S. lividans* TK23 can induce Act production in this organism. Also, we demonstrated in the present study the efficacy of propagating the *S. coelicolor metK* gene in activating the Act production in *S. lividans* TK21 (see Results). Although *S. lividans*, a species closely related to *S. coelicolor* A3(2), carries the entire clusters for Act in its genome, it normally produces less or no Act throughout the whole-cell cycle. Therefore, the propagation of *metK* gene is effective not only for the enhancement of Act production (as seen in *S. coelicolor*) but also for the activation of silent gene(s) (as seen in *S. lividans*), implicating an intrinsic role for SAM syntetase in microbial secondary metabolism. This proposal can be supported by the fact that ND mutation in strain KO-179 was capable of elevating the ability to produce Red and CDA (Fig. 5B and C) in addition to Act.

There are several reports that indicate possible involvement of intracellular SAM in the regulation of antibiotic production (9, 11, 22, 23, 29, 41, 45). Ochi et al. (29) showed previously that exogenous addition of SAM partially restored a defect in the bicozamycin production in certain arginine auxotrophic mutant of *S. griseoflavus*. In addition, the negative effect of methionine on the biosynthesis of antibiotics containing methyl or methoxy groups has been reported (9, 11, 22, 23, 41, 45). Recently, Kim et al. (22) found that the addition of methionine

to the culture medium lowered the production of rapamycin but increased the production of demethylrapamycin in *Streptomyces hygroscopicus*. These authors also revealed that methionine represses the synthesis of methyltransferase, which catalyzes the conversion of demethylrapamycin to rapamycin, in addition to the repression of SAM synthetase itself. A decrease in the level of SAM caused by the repression of SAM synthetase is likely to be responsible for the reduced expression of the methyltransferase. The repressive effect of methionine on the production of other antibiotics may be accounted for by a similar mechanism. However, since the Act (and also bicozamycin) biosynthetic pathway does not contain any steps that require SAM as a methyl donor, the mechanism by which intracellular SAM level affects Act and bicozamycin production is apparently different from the case of rapamycin as described above. The fact that the increased SAM level can activate the transcription of the regulatory gene *act*II-ORF4 for Act production (see below) supports this notion.

The principal regulation of Act production in *S. coelicolor* appears to be the availability of the pathway-specific transcriptional regulatory protein ActII-ORF4, threshold concentration of which is required for efficient transcription of its cognate biosynthetic structural genes. The fact that *metK* overexpression induces transcription of the *act*II-ORF4 is important to note. Several genes have been identified in *S. coelicolor* that are required for both antibiotic production and morphological differentiation (*bld* genes) (5, 7, 15). Also, a number of genes that regulate only antibiotic production have been isolated (1, 2, 6, 10, 24, 33). Certain mutations in these genes were shown to affect (usually reduce) transcription of the *act*II-ORF4 gene (1, 10). Although there is no evidence at present for the existence of a methyltransferase involved in the regulation of antibiotic production, SAM-dependent protein methylation may play a role in controlling the activity of the regulatory proteins that are encoded by such developmental genes. Alternatively, it is possible that DNA or RNA methylation is involved in the expression of these regulatory genes. For example, the extent of methylation of a particular macromolecule (e.g., a particular DNA region) could determine the probability with which a second macromolecule (e.g., a repressor or an activator) can bind to the first, eventually leading to significant alteration of gene expression, in analogy with the known mechanism in *E. coli* (42). Another possibility is that SAM served as a substrate in the biosynthesis of a γ-butyrolactone autoregulator, SCB1 (39, 40), resulting in a stimulation of antibiotic production by *S. coelicolor*. A number of observations in prokaryotic and eukaryotic organisms (8, 25, 35, 42) may guide the search for the methylated molecule that controls initiation of the secondary metabolism onset. Apart from the notion described above that refers to the role of SAM as a methyl donor, SAM might directly be involved in the regulation of Act synthesis as a corepressor or an inducer, as has been demonstrated for methionine regulon expression in *E. coli* (13). It is important to point out that SAM affects morphological differentiation in *S. lividans*, in addition to antibiotic production, in the *metK* gene-propagated cells, as demonstrated by Kim et al. (20). These findings agree with our results that ND mutant KO-179 had a reduced ability to form aerial mycelium (Fig. 5A) and are consistent with the previous work with *B. subtilis* that sporulation of this organism is prevented by SAM at concentrations of

≥ 1 mM (27). It is intriguing to study whether the expression level of regulatory genes for morphological differentiation (e.g., *bldD* and *whiG* in *S. coelicolor* and *spo0A* and *codY* in *B. subtilis*), like that of the *act*II-ORF4 gene, are controlled by the intracellular SAM level or not. We have recently found that another ND mutant KO-132 (*relA str-1*) also overproduces SAM synthetase (unpublished data). KO-132 had been isolated from the *relA* mutant M570 by virtue of low-level streptomycin resistance accompanied by complete restoration of impaired Act production due to the *relA* mutation (28, 37). These results implicate that SAM may exert its effect in a wide variety of biological events by controlling the gene expression in microorganisms as well as eukaryotic cells.

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