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# Modulation of Actinorhodin Biosynthesis in *Streptomyces lividans* by Glucose Repression of *afsR2* Gene Transcription

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**While the biosynthetic gene cluster encoding the pigmented antibiotic actinorhodin (ACT) is present in the two closely related bacterial species, *Streptomyces lividans* and *Streptomyces coelicolor*, it normally is expressed only in *S. coelicolor*—generating the deep-blue colonies responsible for the *S. coelicolor* name. However, multiple copies of the two regulatory genes, *afsR* and *afsR2*, activate ACT production in *S. lividans*, indicating that this streptomycete encodes a functional ACT biosynthetic pathway. Here we report that the occurrence of ACT biosynthesis in *S. lividans* is determined conditionally by the carbon source used for culture. We found that the growth of *S. lividans* on solid media containing glucose prevents ACT production in this species by repressing the synthesis of *afsR2* mRNA; a shift to glycerol as the sole carbon source dramatically relieved this repression, leading to extensive ACT synthesis and obliterating this phenotypic distinction between *S. lividans* and *S. coelicolor*. Transcription from the *afsR2* promoter during growth in glycerol was dependent on *afsR* gene function and was developmentally regulated, occurring specifically at the time of aerial mycelium formation and coinciding temporally with the onset of ACT production. In liquid media, where morphological differentiation does not occur, ACT production in the absence of glucose increased as *S. lividans* cells entered stationary phase, but unlike ACT biosynthesis on solid media, occurred by a mechanism that did not require either *afsR* or *afsR2*. Our results identify parallel medium-dependent pathways that regulate ACT biosynthesis in *S. lividans* and further demonstrate that the production of this antibiotic in *S. lividans* grown on agar can be modulated by carbon source through the regulation of *afsR2* mRNA synthesis.**

The two closely related bacterial species *Streptomyces lividans* and *Streptomyces coelicolor* historically have been distinguished phenotypically by the ability of the latter to produce large amounts of the pigmented antibiotic actinorhodin (ACT), thus generating the deep-blue colonies responsible for the *S. coelicolor* name. While a functional biosynthetic gene cluster encoding ACT is present also in the *S. lividans* chromosome, normally little or no ACT is made. However, introduction of multiple copies of either of two ACT-regulating genes, *afsR* or *afsR2* (7, 12, 13, 22) can circumvent the limitation on ACT production in *S. lividans*, increasing ACT biosynthesis dramatically in this species, as well as in *S. coelicolor* (19, 22, 25). AfsR (13, 22) becomes an active regulator of antibiotic synthesis after it is phosphorylated by the AfsK protein (9, 14), whose gene is located downstream of *afsR* on the chromosome of *S. coelicolor* (18). *afsR2* (25), which is known as *afsS* in *S. coelicolor* (19) and is located immediately 3′ to *afsR*, encodes a 63-amino-acid protein of unknown function. Neither *afsR* nor *afsR2* is absolutely required for ACT biosynthesis, since production of this antibiotic is stimulated by overexpression of either gene in bacteria containing deletions in the other one (7, 25).

Here we report the surprising finding that the phenotypic limitation that historically has distinguished *S. lividans* cultures from those of *S. coelicolor*—namely, the inability of *S. lividans* in its native state to produce significant amounts of ACT from the chromosomal biosynthetic gene cluster—can be remedied simply by changing the culture conditions. We show that ACT biosynthesis in *S. lividans* is repressed by glucose in the media commonly used for cell growth and that a change of carbon source can promote *S. lividans* ACT production by elevating *afsR2* mRNA—increasing cellular pigmentation to a level characteristic of *S. coelicolor* and obliterating this phenotypic distinction between the two *Streptomyces* species.

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## MATERIALS AND METHODS

**Bacterial strains and plasmids.** *S. lividans* TK21, SL94, and SL41 were described previously (25), and a restriction map of the *afsR* and *afsR2* gene region in *S. lividans* is shown in Fig. 1. *S. lividans* SL94-1 is a TK21 derivative containing an *afsR2*::*tsr* segment integrated into the chromosome by targeted gene disruption (described in the section on targeted mutagenesis). *E. coli* DH5α was used as a host to generate DNA for plasmid pMOV96-1. Other organisms and plasmids used in this study were previously described (11, 21, 25).

**Media and culture conditions.** Conditions for *Streptomyces* culture and liquid media (YEME) have been described previously (11). All *Streptomyces* strains were maintained on R5 agar media (11). For evaluation of ACT production, *S. lividans* pregerminated spores were streaked on minimal medium plates containing 0.5% glucose, 0.25% glycerol, or both as a carbon source, followed by 7 days of incubation at 30°C. In liquid cultures, spores were added to a 250-ml baffled flask containing 25 ml of minimal medium that included either glucose or glycerol as the sole carbon source and were then shaken at 30°C for 3 days. Samples (0.5 ml) were taken at various time points and examined for cell density, and the ACT concentrations (11) were determined. All experiments were done at least twice and found to generate consistent results. *Escherichia coli* cultures were grown on Luria-Bertani (LB) agar and liquid broth (21).

**Transformation procedures.** Competent *E. coli* cells and protoplasts of *Streptomyces* were prepared for DNA transformation as described previously (11, 21). Protoplasts of *S. lividans* were regenerated on R5 agar medium for 16 h. Transformants were selected by overlaying the agar surface with 1 ml of sterilized

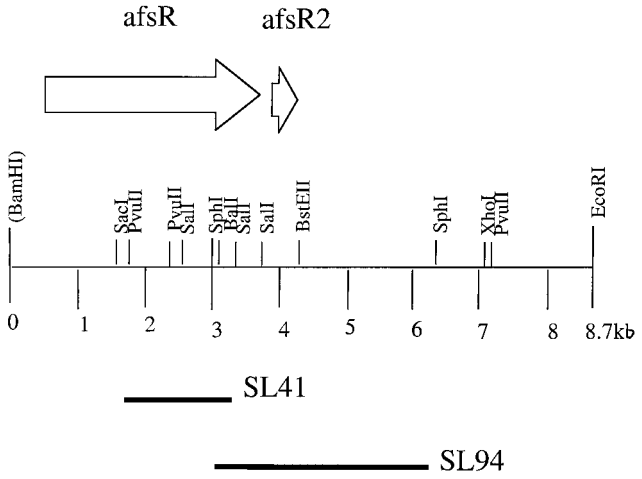


FIG. 1. Restriction map of DNA of the cloned *afsR-afsR2* gene region of *S. lividans* (12). Only relevant restriction endonuclease cleavage sites are indicated. The location and orientation of the translational reading frames encoding AfsR and AfsR2 are shown by open arrows above the restriction map. The locations of chromosomal deletions contained in mutant strains SL41 and SL94 are indicated by closed bars below the map.

water containing 500 μg of thiostrepton. Strains carrying *Streptomyces* plasmids and a mutant strain carrying the *tsr* gene in the chromosome were grown and stored on plates containing 50 μg of thiostrepton per ml. Plates were incubated at 30°C until sporulation occurred and were stored at 4°C.

**PCR.** A *tsr*-containing plasmid construct was generated by PCR with two synthetic oligonucleotide primers containing the *Apa*I site (5′-TGTAGGGCCC AGGCGAATACTTCAT-3′ and 5′-GAGGGGGCCCTCACTGACGAATCG A-3′) using pIJ702 as the DNA template. This PCR product was cloned using the pGEM-T vector system (Promega), and the 1.1-kb *Apa*I fragment of the *tsr* gene was obtained by *Apa*I digestion. Two oligonucleotide primers (5′-TTTTTAAG CTTCGGCGGTGGCCGGGAGCG-3′ and 5′-TTTTTAAGCTTGTCCCCGC GGACGGGGTG-3′) were also designed based on the sequence of the *afsR2* gene in TK21 and used to amplify an *afsR2* construct by PCR. PCR was performed using *Taq* DNA polymerase (Boehringer Mannheim) for 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. The reaction mixture contained PCR buffer (containing 1.5 mM $MgCl_2$), 50 ng of template DNA, 50 μM concentrations of each of the four deoxynucleoside triphosphates, 1 pmol of each primer, and 10% dimethyl sulfoxide.

**Targeted mutagenesis.** For the construction of pMOV96-1 as a suicide vector, pMOV96 (21) was completely digested with *Apa*I and treated with calf intestine alkaline phosphatase (Promega), followed by ligation with a PCR-amplified *Apa*I fragment of *tsr* using T4 DNA ligase (Promega). The pMOV96-1 is an *afsR2*-containing pMOV96 derivative, of which *afsR2* is interrupted at the unique *Apa*I site by the *tsr* gene (see Fig. 3). pMOV96-1 was used to transform protoplasts of *S. lividans* TK21 for disruption of the chromosomal *afsR2* gene. Thiostrepton-resistant transformants, which were presumed to be single crossovers, arose at a frequency of $10^{-3}$ to $10^{-4}$ as determined by analysis of spores developed after 7 days. After several rounds of growth of single crossover transformants on selective media, segregants lacking vector sequences but containing an *afsR2* gene interrupted by a *tsr* insertion (double crossovers) were isolated. One of these was designated SL94-1.

**Southern blotting.** For Southern hybridization, equal amounts of DNA samples were fractionated by electrophoresis in a 0.7% agarose gel and transferred to a positively charged nylon membrane (Amersham) as previously described (8). The DNA concentration was estimated from UV absorbance using the 260/280 nm ratio. Hybridization was carried out in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% *N*-lauroylsarcosine–0.02% sodium dodecyl sulfate (SDS)–1% blocking reagent at 58°C for 16 h. The membrane was washed at high stringency (2× and 0.1× SSC with 0.1% SDS at 68°C). The DNA probe was labeled with a nonradioactive digoxigenin-labeled dUTP (Boehringer Mannheim).

**S1 nuclease protection analysis and Northern blotting.** The isolation of total RNA from *Streptomyces* and Northern blot hybridization were described in detail elsewhere (11). RNA was isolated from *S. lividans* plate cultures at the following three separate phases of growth on minimal agar plates using either glycerol or glucose as the sole carbon source: substrate mycelium, aerial mycelium, and spores. Cells were scraped from cellophane disks (8). The RNA concentration was normalized based on UV absorbance at 260 nm and verified by determining the amount of 5S RNA. The $^{32}$P-labeled DNA fragment used as a Northern blot hybridization probe was the same as that used for S1 mapping and was randomly labeled using a hexanucleotide priming kit (Amersham). The probe used to map the *afsR2* promoter was generated using PCR from *S. lividans* TK21 total DNA as template using a 5′-end-labeled oligonucleotide primer internal to the *afsR2* gene (*afsR2*II; 5′-TCCATCGTGGTGATCGCTTCGTT-3′) and an unlabeled oligonucleotide (*afsR2*I; 5′-TCGACCGGCGGTGGCCGGGAGCGTT-3′). AfsR2II was labeled using [γ-$^{32}$P]ATP (3,000 Ci mmol$^{-1}$; DuPont-NEN) (14). For this assay, 40 μg of RNA and 25 fmol of the probe were resuspended in 20 μl of sodium-trichloroacetic acid butter, hybridized at 45°C overnight following initial denaturation at 65°C for 15 min, and digested with S1 nuclease. RNA-protected fragments were resolved on a 6% polyacrylamide sequencing gel.

## RESULTS

**Carbon-source-regulated *afsR2*-dependent stimulation of ACT production in *S. lividans*.** Since *afsR2 (afsS)* overproduction from multiple gene copies borne by plasmids can induce *S. lividans* to synthesize large amounts of ACT, we sought to identify conditions that upregulate a single chromosomal copy

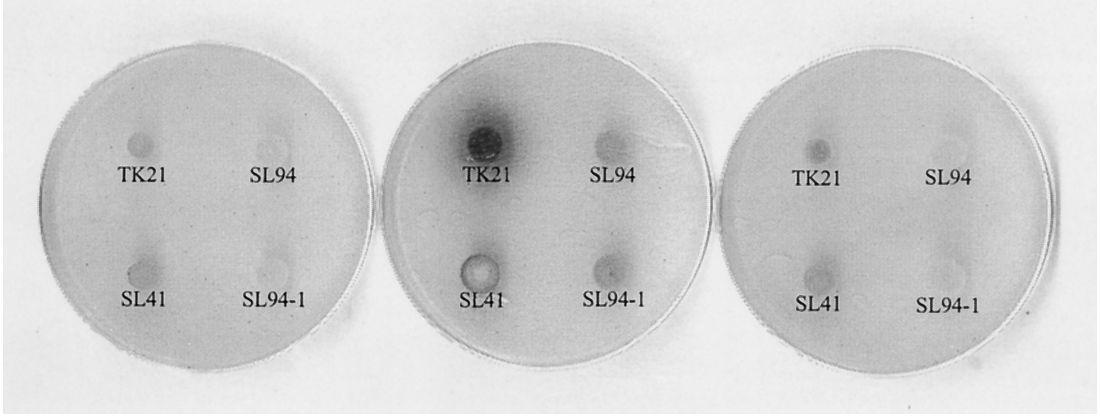


FIG. 2. ACT production by *S. lividans* (TK21) in the presence of glycerol. Portions (10 μl) of diluted spore stocks of *S. lividans* TK21, SL41, SL94, and SL94-1 were spotted onto minimal-agar plates containing 0.5% glucose (left), 0.25% glycerol (middle), or both (right) as a carbon source. After a week of incubation at 30°C, each plate was placed upside down for ammonia fuming as well as for photography.

of *afsR2* using visually determined elevation of ACT biosynthesis as an assay. During these experiments we observed that ACT production by *S. lividans* TK21 (*afsR2*$^+$), which normally is not observed during growth on minimal agar, even after an extended incubation period, when glucose is the carbon source (Fig. 2, left), occurred in large amounts when glycerol was substituted as the sole carbon source (Fig. 2, middle). The ability of TK21 to produce ACT in glycerol-containing media was reversed when glucose was also present (Fig. 2, right), indicating that ACT synthesis in the strain is subject to glucose repression. The TK21-derived *S. lividans* strain SL94, which lacks DNA segment of ca. 3.5 kb that includes both the *afsR2* gene and the sequences adjacent to it (Fig. 1) (25), showed only limited ACT biosynthesis in all of these media (Fig. 2), suggesting that the ACT production observed during growth in glycerol requires genetic information absent in this strain.

The deletion in SL94 includes a small C-terminal segment of *afsR* and all of *afsR2* (Fig. 1). Since the C-terminal segment is not required for *afsR* activity (13), we tentatively attributed the inability of SL94 to effectively synthesize ACT during growth in glycerol to the deletion of *afsR2*. To confirm this presumption, we used targeted gene disruption to specifically inactivate the chromosomal *afsR2* locus in TK21; this was done using a suicide vector, plasmid pMOV96-1 (25), in which the cloned *afsR2* gene is interrupted by the thiostrepton resistance gene (*tsr*). Following selection of thiostrepton-resistant transformants, a randomly selected clone (SL94-1) was analyzed by PCR and Southern blotting to confirm the absence of the vector and also the insertion of *tsr* specifically in the chromosomal *afsR2* gene (Fig. 3). On minimal-medium plates containing glycerol as the sole carbon source, SL94-1 showed results identical to those observed for SL94 (Fig. 2, middle), indicating that stimulation of ACT synthesis in *S. lividans* upon relief from glucose repression specifically requires *afsR2*.

**Expression of *afsR2* is affected by carbon source, morphological differentiation, and the *afsR* gene.** As already noted, introduction of multiple plasmid-borne copies of the *afsR2* gene can stimulate ACT biosynthesis in *S. lividans* (25). Our discovery that *S. lividans* TK21, which lacks multiple copies of *afsR2*, can produce large amounts of ACT when cultured on media containing glycerol but lacking glucose (Fig. 2), together with the finding that such stimulation of ACT production is dependent on *afsR2*, suggested that carbon source regulation of ACT biosynthesis may be mediated through the control of *afsR2* expression. This notion was tested directly by Northern blot analysis that compared steady-state levels of *afsR2* mRNA in TK21 and SL94 cells grown on minimal medium plates containing either glucose or glycerol as the sole carbon source. As seen in Fig. 4, *afsR2* transcripts of ~400 nucleotides nt were observed in TK21 when grown in glycerol but not when grown in glucose and were not detected in total RNA isolated from the SL94 mutant strain. Additionally, a high steady-state level of *afsR2* mRNA was seen only at the time of onset of both aerial mycelium formation and ACT production (day 4 of the cycle), indicating the developmental regulation of carbon source-mediated *afsR2* transcription. S1 protection analysis (Fig. 4B) using an *afsR2*-specific probe identified the site of initiation of the carbon source-regulated transcripts, confirming that the transcripts shown by Northern blotting to be present at the onset of aerial mycelium formation are initiated at the *afsR2* promoter.

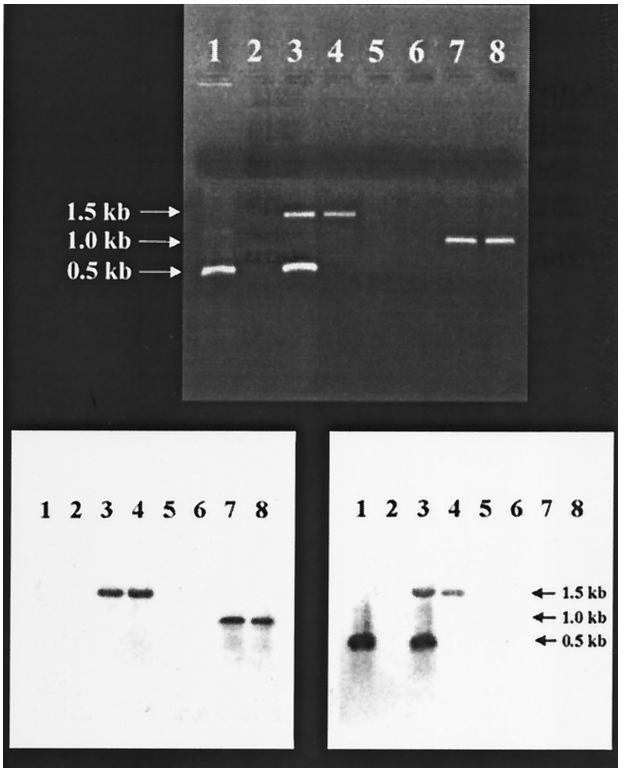


FIG. 3. (Top) Agarose gel electrophoresis of the PCR analysis with *afsR2* (lanes 1 to 4) and *tsr* (lanes 5 to 8) primers. Each lane depicts a PCR result using as template the indicated chromosomal DNA. Lanes 1 and 5, TK21 chromosomal DNA; lanes 2 and 6, SL94 chromosomal DNA; lanes 3 and 7, single-crossover TK21 chromosomal DNA; lanes 4 and 8, double-crossover TK21 (SL94-1) chromosomal DNA. The single band shown in lane 1 is the *afsR2* (~500-bp) PCR fragment corresponding to the chromosomal copy of *afsR2* in TK21. The absence of amplification products in lanes 2 and 5 reflects the absence *afsR2* and *tsr* in the chromosome of SL94. Lane 3 shows two bands: the large one is the PCR product corresponding the *tsr*-interrupted *afsR2* (ca. 1.5 kb), and the small one is the intact *afsR2*. The one band seen in lane 4 is *tsr*-interrupted *afsR2* resulting from the double crossover. Both lanes 7 and 8 show PCR products of *tsr* secondary to the insertion of *tsr* in the chromosomal copy of *afsR2* in TK21. (Bottom) Southern blot hybridization analysis of PCR products using *tsr* (left) or *afsR2* (right) probe DNA. The band sizes are indicated by the arrows. Lanes are as indicated in the top panel.

During these studies we observed that ACT biosynthesis also did not occur in the *S. lividans afsR* deletion mutant, SL41, even when grown in glycerol in the absence of glucose (Fig. 2 and 4). However, since *S. lividans* cells containing multiple copies of *afsR2* but mutated in *afsR* can produce large amounts of ACT (25), ACT biosynthesis cannot have an absolute requirement for *afsR*. Taken together with the findings described above, the absence of *afsR2* transcripts in strain SL41 (Fig. 4) suggests that *afsR*, while not needed for ACT production per se in *S. lividans*, stimulates the transcription that occurs under normal conditions from the chromosomal *afsR2* locus during growth on media containing glycerol.

**ACT production in liquid cultures is independent of the AfsR2/AfsR pathway.** While streptomycetes differentiate both

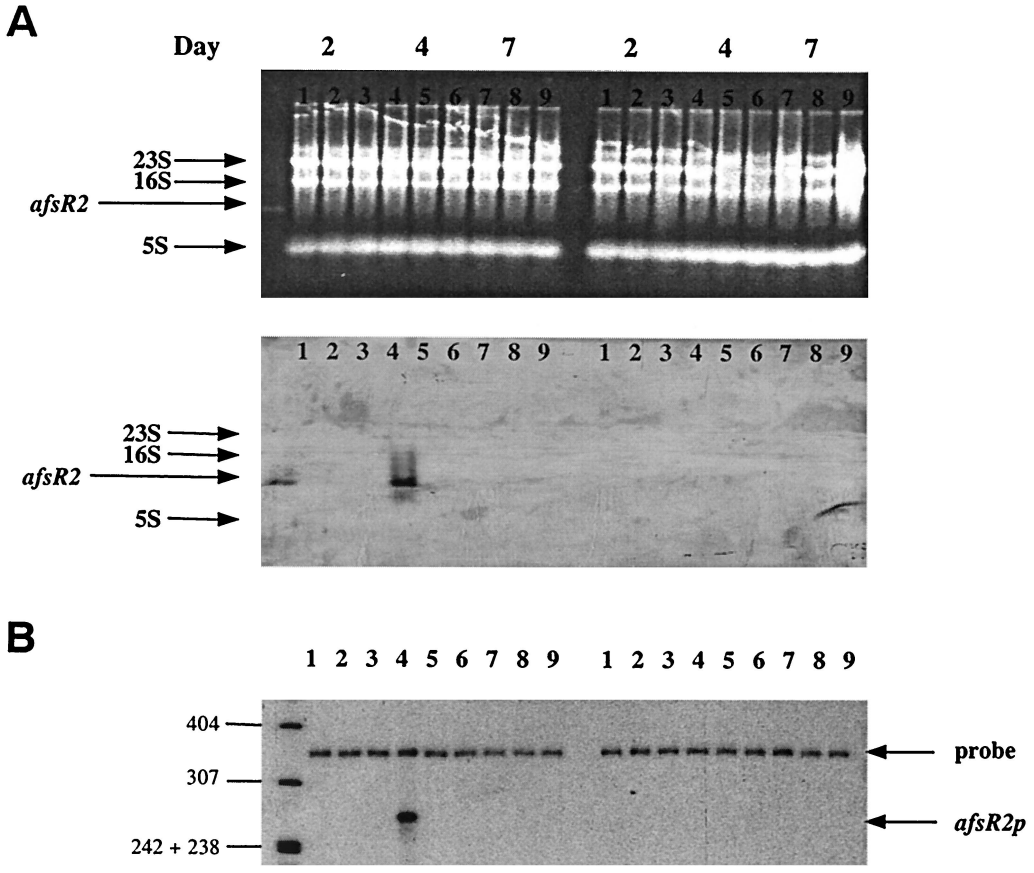


FIG. 4. (Top) Northern blot analysis of total RNA isolated from *S. lividans* TK21 (lanes 1, 4, and 7), SL94 (lanes 2, 5, and 8), and SL41 (lanes 3, 6, and 9) grown on minimal-medium plates containing either glycerol (left) or glucose (right) as the sole carbon source. Total RNAs from the three strains were isolated at day 2 (substrate mycelia; lanes 1, 2, and 3), day 4 (aerial mycelia; lanes 4, 5, and 6), and day 7 (spores; lanes 7, 8, and 9). The top panel, which indicates equal loading of samples into lanes, shows the ethidium bromide-stained gel, indicating the locations of rRNA. The location of purified *afsR2* mRNA isolated by prehybridization with *afsR2* probe DNA which was the same as that used for S1 mapping (lane at extreme left). The lower panel shows the Northern blot. (Bottom) S1 nuclease protection analysis of transcription of *afsR2* during development occurring in surface-grown cultures of *S. lividans* TK21 (lanes 1, 4, and 7), SL94 (lanes 2, 5, and 8), and SL41 (lanes 3, 6, and 9). RNA was isolated from cells grown on cellophane-covered minimal-medium plates containing either glycerol (left) or glucose (right) as the sole carbon source for 2 days (substrate mycelia; lanes 1, 2, and 3), 4 days (aerial mycelia; lanes 4, 5, and 6), and 7 days (spores; lanes 7, 8, and 9). The uniquely 5′-end-labeled probes were prepared as described in Materials and Methods. A protected 282-bp fragment representing *afsR2* transcripts detected only in *S. lividans* TK21 (*afsR2*$^+$) grown on glycerol minimal-medium plates for 4 days (lane 4). The position of DNA size marker bands is shown to the left of the figure.

morphologically and physiologically, the two processes are independent (3). *Streptomyces* species commonly do not complete their morphological development or sporulate in liquid cultures but nevertheless synthesize the same antibiotics and other secondary metabolites that they produce on solid media during the formation of aerial mycelia and spores (3). Several pleiotropic loci that govern antibiotic production have been identified; some of these affect only antibiotic production, whereas others affect both antibiotic production and morphological differentiation, suggesting that the two processes share elements of genetic control (3, 4). To learn whether the life cycle-associated increased *afsR2* mRNA expression observed in cells cultured on solid media requires morphological differentiation per se, we examined the effect of carbon source on ACT production in cells grown in liquid. As shown in Fig. 5, strains TK21, SL94, and SL41 cultured in liquid minimal media (11) containing glucose as the sole carbon source all failed to produce ACT, as had been observed for solid medium. When glycerol was substituted for glucose as the carbon source, strain TK21, which contains functional *afsR* and *afsR2* genes, produced copious amounts of ACT, as had also occurred during growth on solid medium lacking glucose. Surprisingly however, we observed that, unlike plate-grown cells, the *S. lividans* strains utilizing glycerol as the sole carbon source also produced ACT efficiently in liquid medium when they carried deletions in *afsR* or *afsR2*, indicating that ACT biosynthesis had occurred by a pathway that is independent of both of these genes. Consistent with this conclusion was our finding that the growth of TK21 cells in glycerol-containing liquid media showed ACT synthesis in the absence of detectable *afsR2* mRNA (Fig. 6).

Additional evidence that *afsR2* mRNA production does not govern ACT production in liquid media was provided by the observation that cells grown in liquid media containing glucose as the sole carbon source failed to produce ACT, despite their ability to synthesize normal-size *afsR2* transcripts (Fig. 6).

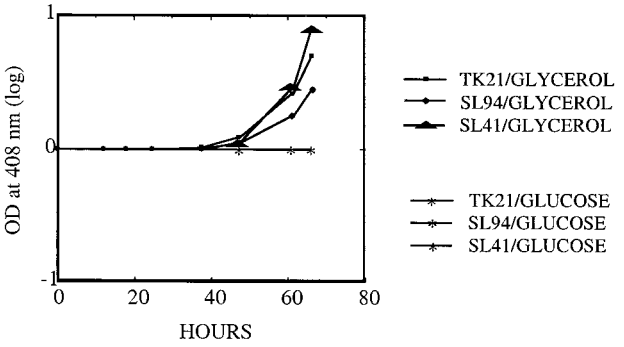


FIG. 5. Actinorhodin production by *S. lividans* (TK21, SL94, and SL41) in liquid culture. Each *S. lividans* was inoculated into a 250-ml baffled flask containing 25 ml of minimal medium that included either glucose or glycerol as a sold carbon source, followed by incubation at 30°C for 3 days with shaking. Samples (0.5 ml) were taken at various time points and measured.

Thus, under the liquid medium conditions we employed, regulation of the carbon source-dependent growth we observed for ACT biosynthesis in *S. lividans* may occur at a point downstream of the action of *afsR2* or by an independent pathway. Interestingly, however, cells grown in liquid media containing glycerol produced high-molecular-weight transcripts detected by the *afsR2* probe (Fig. 6, lane 1), raising the alternative possibility that *afsR2* mRNA in such cells may be initiated or terminated at a different location.

## DISCUSSION

*S. lividans* has been widely studied for more than 20 years and has been used extensively as a host for DNA cloning in studies of *Streptomyces* biology. While *S. lividans* contains the complete biosynthetic pathway for ACT biosynthesis, this *Streptomyces* species has long been thought to be incapable of synthesizing significant amounts of ACT unless stimulated to do so by the introduction of multiple copies of regulatory genes such as *afsR* and *afsR2*. The data presented here show that in *S. lividans* the choice of the carbon source is crucial to *afsR2* expression and consequently to ACT biosynthesis when only a single copy of *afsR2* is present. During a search for conditions that upregulate expression of a single chromosomal copy of *afsR2*, we found that ACT biosynthesis is subject to glucose repression during growth on solid media and that the use of glycerol as the sole carbon source enables *S. lividans* to produce the copious amount of deep-blue ACT pigment that has given *S. coelicolor* its species name (19, 22, 25). This carbon source-dependent activation of ACT production is mediated through induction of mRNA synthesis initiated at the *afsR2* promoter, and it requires *afsR*. As derepression of the *afsR2* promoter during normal growth on solid media coincides temporally with the onset of the production of both aerial mycelia and ACT (Fig. 4), the turning on of ACT biosynthesis during morphological differentiation may occur at least in part by regulation at the level of *afsR2* mRNA production.

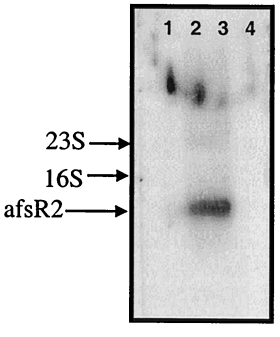


FIG. 6. Northern blot analysis of total RNA isolated from *S. lividans* grown for 3 days 30°C in minimal liquid culture. Lane 1, TK21 cultured with glycerol; lane 2, TK21 cultured with glucose; lane 3, TK21 cultured with both; lane 4, SL94 with glycerol.

Our studies also indicate the existence of a still-undefined separate carbon source-regulated ACT biosynthetic pathway that operates in *S. lividans* during growth in liquid media and is independent of both *afsR* and *afsR2* (see also reference 3). Interestingly, the *afsR2*-dependent and *afsR2*-independent mechanisms of carbon source regulation of ACT synthesis in *S. lividans* have the same ultimate effect on ACT production despite their different effects on *afsR2* expression. Whereas the *afsR* and *afsR2* genes individually are not essential for the synthesis of ACT, multiple plasmid-borne copies of either the *afsR* or the *afsR2* gene can stimulate ACT production in *S. coelicolor* and *S. lividans* species independently of the carbon source. Potentially, this stimulation may occur by a direct gene dosage effect on protein production or by a mechanism in which the copies of *afsR2* exceed the capacity of a repressor of *afsS*. Whether the effect of a high *afsR2* copy number is direct or occurs by activation of another regulator is not known.

Unlike *S. lividans, S. coelicolor* normally can produce large amounts of ACT during growth in glucose (7), suggesting either that AfsR2 synthesis in *S. coelicolor* is not sensitive to glucose repression or, alternatively, that ACT production on solid medium by this species does not depend on the activation of *afsS* (the *S. coelicolor* homolog of *afsR2* [19]). Nevertheless, *afsR2* production in *S. coelicolor*, like *afsR2* production in *S. lividans*, requires expression of *afsR* (7).

Earlier experiments have shown the effects of carbon, source, nitrogen, phosphate, and other culture medium variables on antibiotic production in *Streptomycetes* (1, 2, 6, 17), and glucose repression of a variety of streptomycete promoters is known to occur (15, 16, 20, 23). The investigations reported here provide specific evidence for the physiological control of *S. lividans* regulatory genes that affect the expression of ACT. Accordingly, they raise the prospect that antibiotics apparently not synthesized by particular species under commonly used laboratory growth conditions may be under control of antibiotic biosynthetic regulatory genes that are subject to carbon source repression.

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E.-S.K. and H.-J.H. contributed equally to this work.

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