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Activation of Antibiotic Biosynthesis by Specified Mutations in the *rpoB* Gene (Encoding the RNA Polymerase β Subunit) of *Streptomyces lividans*

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We found that the biosynthesis of actinorhodin (Act), undecylprodigiosin (Red), and calcium-dependent antibiotic (CDA) are dramatically activated by introducing certain mutations into the *rpoB* gene that confer resistance to rifampin to *Streptomyces lividans* 66, which produces less or no antibiotics under normal growth conditions. Activation of Act and/or Red biosynthesis by inducing mutations in the *rpoB* gene was shown to be dependent on the mutation's position and the amino acid species substituted in the β-subunit of the RNA polymerase. Mutation analysis identified 15 different kinds of point mutations, which are located in region I, II, or III of the *rpoB* gene and, in addition, two novel mutations (deletion of nucleotides 1287 to 1289 and a double substitution at nucleotides 1309 and 1310) were also found. Western blot analyses and S1 mapping analyses demonstrated that the expression of *act*II-ORF4 and *redD*, which are pathway-specific regulatory genes for Act and Red, respectively, was activated in the mutants able to produce Act and Red. The ActIV-ORF1 protein (an enzyme for Act biosynthesis) and the RedD protein were produced just after the upregulation of ActII-ORF4 and RedZ, respectively. These results indicate that the mutation in the *rpoB* gene of *S. lividans*, resulting in the activation of Act and/or Red biosynthesis, functions at the transcription level by activating directly or indirectly the key regulatory genes, *act*II-ORF4 and *redD*. We propose that the mutated RNA polymerase may function by mimicking the ppGpp-bound form in activating the onset of secondary metabolism in *Streptomyces*.

Members of the genus *Streptomyces* produce most of the natural product antibiotics used clinically today. The activation of antibiotic production, often coupled to morphological development, involves many different pathways in the same organism (reviewed by Chater [8]; also see reference 35 for a brief review). Multiple and coordinated regulatory mechanisms controlling antibiotic biosynthesis are still poorly understood. *Streptomyces coelicolor* A3(2), the best genetically studied streptomycete, produces four biochemically and genetically distinct antibiotics: actinorhodin (Act), undecylprodigiosin (Red), methylenomycin, and calcium-dependent antibiotic (CDA) (reviewed by Hopwood et al. [19] and Chater and Bibb [10]). The gene clusters responsible for the production of Act, Red, methylenomycin, and CDA have been cloned and characterized (9, 12, 15, 29). Linked to these gene clusters, various pathway-specific regulatory genes (*act*II-ORF4, *redD*, and *redZ*, etc.) have been identified, and among these *act*II-ORF4 and *redD* have been shown to regulate the Act and Red biosynthesis genes, respectively. Transcription of *redD* has in turn been shown to be regulated by another regulatory gene, *redZ* (15, 31, 43). These results taken together indicate that these pathway-specific regulatory genes act as positive regulators for their respective biosynthesis genes. For example, strains carrying mutant genes that fail to accumulate regulatory gene transcripts also fail to cosynthesize Act or Red. Moreover, upregulation of *act*II-ORF4 and *redD* resulted in the upregulation of Act and Red, respectively (4, 13). In addition, many pleiotropic regulatory genes (e.g., *absA*, *absB*, *afsR*, *afsR2*, and *abaA*) in *S. coelicolor* have been found to regulate antibiotic biosynthesis (2, 14, 20).

Streptomyces lividans 66, a species closely related to *S. coelicolor* A3(2), carries the entire clusters for Act and Red in its genome but normally produces less or no Act and Red throughout the whole-cell cycle, although this strain is known to produce abundant amounts of their pigmented antibiotics under certain growth conditions. Recent studies revealed that the *cutRS* signal transduction system and LysR-type transcriptional regulator negatively regulate Act biosynthesis in *S. lividans* (7, 30). It was found that *afsR2*, which encodes a 63-amino-acid protein, stimulates Act and Red production in *S. lividans* (27, 42). How to activate this silent antibiotic biosynthetic gene cluster can give some clues about the regulation system for antibiotic production. We previously reported that introduction of the *str* mutation, which confers resistance to streptomycin, could activate Act production in *S. lividans* (38). Introducing the *str* mutation into other *Streptomyces* species was also effective in improving antibiotic production (21). Moreover, the *str* mutation could also suppress the detrimental effect of *relA* and *relC* on antibiotic production due to the failure to accumulate ppGpp (34, 38). Recently, we found that the acquisition of resistance to rifampin confers the ability to produce Act in *relA* and *relC* mutants of *S. coelicolor*, which were both defective in Act production due to *rel* mutations (J. Xu, Y. Tozawa, and K. Ochi, unpublished data). In a previous study (22), we demonstrated that Act biosynthesis could be

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TABLE 1. Screening and characterization of *rif* mutants of *S. lividans* 66

Strain[a]	Relevant genotype	Frequency of mutants with the same mutation (no. with mutation/total tested)	Resistance level (μg/ml) to rifampin[b]	Production of antibiotics in R4 liquid culture	
				Red (mg/g)[c]	Act (OD_{633})
S. lividans 66	Prototrophic wild type		10	0.84	0.08
HY-1	*rif-1*	8/60	400	0.79	0.53
HR-1	*rif-2*	3/60	400	0.36	0.17
HC-1	*rif-3*	2/60	400	1.39	0.08
HN-1	*rif-4*	1/60	400	1.28	0.09
HL-1	*rif-5*	2/60	400	0.95	0.23
CT-1	*rif-6*	7/60	200	1.20	0.18
NY-1	*rif-7*	4/60	400	1.07	0.05
LP-1	*rif-8*	1/60	300	0.40	0.02
EN-1	*rif-9*	2/60	400	4.36	0.08
DV-1	*rif-10*	2/60	400	2.03	0.35
DE-1	*rif-11*	4/60	300	0.85	0.24
DN-1	*rif-12*	2/60	400	1.05	0.12
DG-1	*rif-13*	3/60	400	0.82	0.08
SL-1	*rif-14*	2/60	400	0.48	0.16
SP-1	*rif-15*	1/60	300	0.68	0.73
RH-1	*rif-16*	3/60	400	1.03	0.15
RC-1	*rif-17*	3/60	300	0.86	0.38

[a] All mutant strains isolated in this study were spontaneously generated rifampin-resistant mutants from *S. lividans* 66.
[b] Determined after 4 days of cultivation on GYM agar.
[c] That is, one milligram of Red per gram of dry mycelia.

enhanced when a *rif* mutation (conferring resistance to rifampin) is introduced into the wild-type strain of *S. coelicolor* A3(2). A variety of mutations in the *rpoB* gene, which encodes the RNA polymerase β-subunit, are known to confer resistance to rifampin. Here, we report that certain mutations, when introduced into the RNA polymerase β subunit, can activate the genes for antibiotic production in *S. lividans*. The mechanism for this activation was investigated by Western blotting and S1 mapping analysis.

MATERIALS AND METHODS

Bacterial strains and preparation of mutants. The wild-type strain 1326 of *S. lividans* 66 and the rifampin-resistant mutants used in this study are listed in Table 1. Spontaneous rifampin-resistant (*rif*) mutants were obtained from colonies that grew within 7 days after wild-type spores were spread on GYM agar containing 100 or 200 μg of rifampin/ml. The mutants used for subsequent study were selected after single-colony isolation was performed.

Media and culture conditions. GYM, R4, ONA, ONB, and SMMS media were described previously (26, 32, 38). Culture conditions were as reported previously for *S. coelicolor* (22). Determination of antibiotic productivity was always performed by using triplicated culture flasks, and the mean values of the three samples were presented in Table 1 and Fig. 2. The reproducibility of the results was confirmed at least by two separate experiments (for Table 1 and Fig. 2 to 4).

Assays for antibiotics. Act was assayed as previously described (22). When cultivation was carried out with GYM medium, 10 ml of 1 N KOH was added to every 100 ml of culture broth. After standing for 1 h at room temperature, the culture was filtered, and the optical density at 633 nm (OD_{633}) of each filtrate was determined. For Red, 100 ml of culture broth was filtered, and the mycelial pellet was washed and dried under vacuum conditions. After being weighed, the dried mycelia were first extracted with 0.1 N KOH to dissolve the Act and then extracted with methanol (adjusted to pH 2) overnight at room temperature. The amount of Red was determined by measuring the OD_{530} (ε_{530} = 100,500) (26). The CDA was assayed according to the method of Kieser et al. (26). Then, 5 μl of spore suspension (containing ca. 10^5 spores) was spotted on R4 or SMMS agar plates, followed by incubation at 30°C for 48 h. Oxoid nutrient broth (10 ml) was inoculated with *Staphylococcus aureus* 209P, and the mixture was incubated at 30°C with shaking until the OD_{650} reached 0.7 to 0.8. For each culture plate, 0.5 ml of the *S. aureus* culture was added to 10 ml of soft nutrient agar (5 ml of Oxoid nutrient agar plus 5 ml of Oxoid nutrient broth) with or without 60 mM $Ca(NO_3)_2$. The mixture was used to overlay the *S. lividans* cultures. CDA production was detected as an inhibition zone of *S. aureus* after an overnight incubation at 30°C.

Mutation analysis of the *rpoB* gene. The *rpoB* gene fragments in *S. lividans* 66 and the *rif* mutants were amplified by PCR by using the appropriate genomic DNA as templates, and the design of the synthetic oligonucleotide primers was based on the sequence for the *S. coelicolor* M145 *rpoB* gene (GenBank accession no. AL160431). Primers P1 (forward, 5′-CCGAGTTCACCAACAACGAGAC C-3′) and P2 (reverse, 5′-CGATGACGAAGCGGTCCTCC-3′) were used to amplify a 1.2-kb fragment from nucleotides (nt) 374 to 1582. Primers P1, P2, and P3 (forward, 5′-GGCCGCTACAAGGTGAACAAGAAG-3′) were used as sequencing primers. Another fragment (nt 1453 to 2154) was amplified with primers P4 (forward, 5′-TCGCTCGCCTCGTACGGC-3′) and P5 (reverse, 5′-CTC GTAGTTGTGACCCTCCC-3′). PCR was performed with *Taq* polymerase (Takara LA-*Taq*) according to the manufacturer's instructions. A Perkin-Elmer-Cetus thermal cycler was used, and conditions were as follows: 5 min of preincubation at 96°C; followed by 30 cycles of 96°C for 0.3 min, 55°C for 0.2 min, and 72°C for 0.5 min; and final step at 72°C for 10 min. PCR products were directly sequenced by the dideoxy chain termination procedure by using the BigDye Terminator Cycle Sequencing kit (Perkin-Elmer/Applied Biosystems, Foster City, Calif.).

Western blot analysis. Antibodies against S12, ActII-ORF4, ActIV-ORF1, RedZ, and RedD proteins were prepared by injecting purified recombinant proteins into rabbits intraperitoneally. After purification with conventional methods, these antibodies were used as primary antibodies at a dilution of 1:3,000, as described previously (24). Details of this antibody preparation will be reported elsewhere. The enhanced chemiluminescence Western blotting detection system for chemiluminescent detection was used as specified by the manufacturer (Amersham Pharmacia Biotech).

S1 nuclease protection assays. RNA was extracted from cultures as described by Kieser et al. (26) by using Kirby method. For each S1 nuclease reaction, 50 μg of RNA was dried down with the appropriate ^{32}P-labeled DNA probes (ca. 30,000 cpm) and hybridized overnight at 45°C in sodium-trichloroacetic acid buffer after denaturation at 65°C for 15 min. S1 nuclease digestions and analyses of RNA-protected fragments were performed as described previously (4). Uniquely end-labeled probes were generated by PCR as follows. For *act*II-ORF4, the primer 5′-GGTCCGCCCACAACTCCTC was labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase with the unlabeled primer 5′-GCCG TATCAGGAATGCCAGA and 1 μg of genomic DNA as a template. PCR conditions consisted of 30 cycles of 30 s at 95°C, 30 s at 58°C, and 50 s at 72°C in the presence of 5% glycerol. The resultant probe was 368 bp, which gave a protected fragment of 190 nt in S1 nuclease protection experiments. Probes for *redZ* and *redD* were made in a similar way but with the different set of primers. For *redZ*, labeled primer 5′-CCCAATATGTTGATTTCCACGC, unlabeled

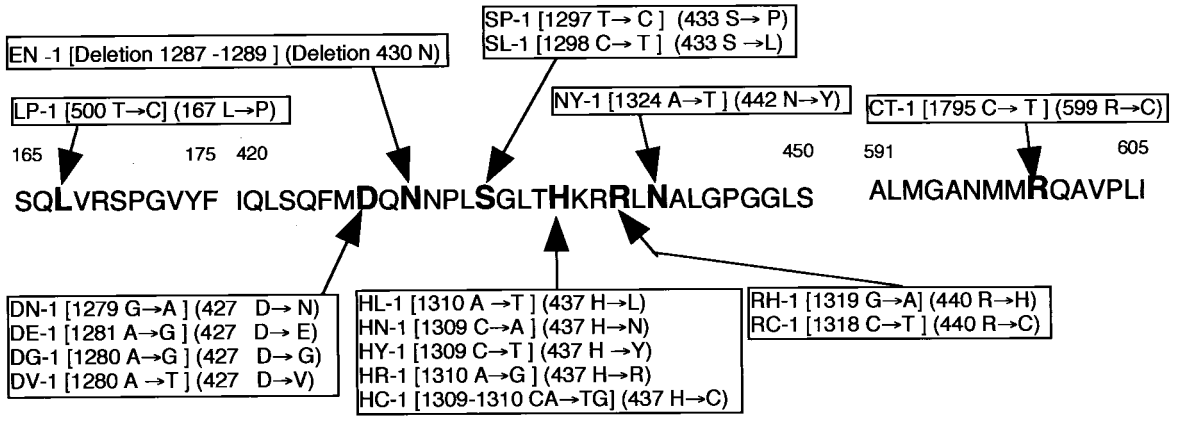

FIG. 1. Nucleotide changes detected in *rpoB* gene and the corresponding amino acid alterations in the β-subunit of RNA polymerase in the *S. lividans rif* mutants. Numbering originates from the starting amino acid (Met) of the open reading frame. The mutation positions are indicated by arrows. Strain numbers, nucleotide changes, and amino acid changes are denoted in this order in boxes.

primer 5′-CTTCGTTTGCGTCGTTCAGTT and genomic DNA as a template produced a 367-bp product that yielded a protected fragment of 217 nt. For *redD*, labeled primer 5′-CACCAGTTCTTCGACCGACG and unlabeled primer 5′-AAGCCCCTCTCCAAGTGTGC produced a 480-bp product that yielded a protected fragment of 210 nt. Control experiments in which 50 μg of yeast tRNA replaced experimental total RNA samples were performed with each set of S1 nuclease protection assays to confirm that there were no signals. All of the S1 nuclease protection experiments were carried out twice with RNA isolated from independently cultured cells to ensure the reproducibility of the results.

Complementary experiment. A 5.0-kb fragment from the *Not*I and *Bam*HI digest of cosmid SCD82 (GenBank accession no. AL160431) that contains the intact *rpoB* gene of *S. coelicolor* M145 was cloned into the pV1 [a low-copy plasmid in *Streptomyces* spp. consisting of a pIJ941 sequence and a pBluescript SK(+) sequence (24)]. The replicative ligations were transformed into *S. lividans rif* mutants (EN-1 and SL-1). *Streptomyces* plasmids preparation and transformation were carried out as described by Kieser et al. (26).

RESULTS

Screening and mutation analysis of the *rif* mutants. We first attempted to isolate a number of *rif* mutants, which developed spontaneously. When the spores of *S. lividans* 66 were spread and incubated on GYM agar containing 100 or 200 μg of rifampin/ml, spontaneous *rif* mutants developed after 4 to 7 days at a frequency of 10^{-7} to 10^{-9}. Sixty mutants were randomly selected and examined for the production of Act and Red by using R4 and GYM media. Strikingly, >50% of the mutants tested exhibited a significantly elevated ability to produce Act and/or Red compared to the wild-type strain. In contrast, nearly 40% of the mutants showed the same or a reduced ability to produce Act and/or Red (data not shown). All of the mutants tested demonstrated a high level (20- to 40-fold) of resistance to rifampin (Table 1).

There is strong evidence that shows rifampin resistance frequently results from a mutation in the *rpoB* gene, which encodes the β-subunit of RNA polymerase (23, 39, 44). We therefore sequenced the *rpoB* gene from the 60 mutant isolates and compared them to the wild-type strain. Since all *rif* mutations in the *rpoB* gene found thus far are known to be located in three conserved regions, we focused on only three: region I covering nt 370 to 600, region II covering nt 700 to 1500, and region III covering nt 1600 to 2100. The sequencing data revealed that the vast majority (38 of 60) of the *rif* mutants possessed a point mutation in region II, 7 mutants were found with a point mutation in region III, and only 1 was found with a mutation in region I (Fig. 1). No mutation was detected in the *rpoB* gene of 10 *rif* mutant isolates, which were not listed in Table 1. In addition to point mutations, we found a deletion mutation (deletion of nt 1287 to 1289, resulting in the deletion of Asn-430) and a double substitution mutation (substitution of nt 1309 and 1310, resulting in an amino acid alteration at position 437). Thus, by analyzing 60 *rif* mutants we detected ultimately 15 kinds of point mutations at seven distinct positions, plus one deletion and one substitution mutation (Fig. 1). Among these mutations, changes of Leu-167 to Pro, His-437 to Cys, and the deletion of Asn-430 are novel *rif* mutations.

Activation of antibiotic biosynthesis by *rif* mutations. (i) Act production. Although *rif* mutations occur in the *rpoB* gene and result in a high-level resistance to rifampin, only specific mutations could elicit *S. lividans* to produce Act as shown when examined in liquid culture by using R4 (Table 1) and GYM (Fig. 2) media. It is notable that the *rif* mutants which have the same mutation in *rpoB* (for example, all eight isolates in *rif-1* type have the identical mutation; see Table 1) showed a similar phenotype with respect to antibiotic production. Further evidence for a causal relationship came from a complementary experiment (see Materials and Methods). By introducing the wild-type *rpoB* gene into mutant EN-1 (*rif-9*) or SL-1 (*rif-14*), the impaired sporulation and overproduction of Red or Act were both restored to the level of the parental (wild-type) strain (data not shown). These results indicate that the *rif* mutations detected are responsible for the observed changes in phenotype as presented in Table 1, where only the representative mutant strains are listed. Also, it is important to note that Act productivity depends not only on the mutational position but also on the amino acid species altered at that position. The mutants SL-1 (*rif-14*) and SP-1 (*rif-15*), which altered Ser-433, exhibited the highest Act productivity when cultured in GYM and R4 media, respectively. We then examined the mutants to see whether or not they could produce high levels of Act in solid culture. Our results show that, except for a couple of mutants (SP-1 and SL-1), almost all of the mutants

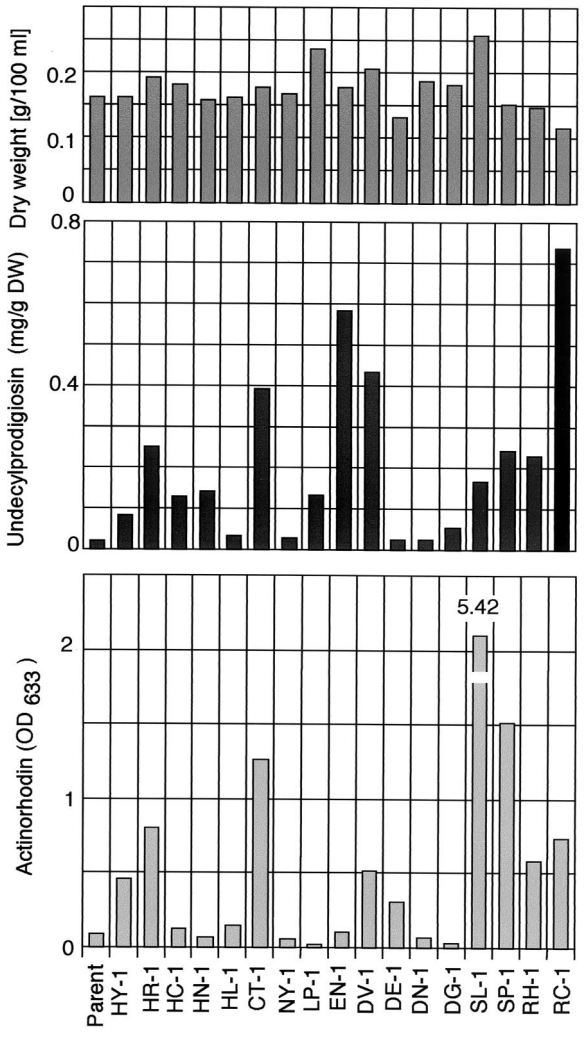

FIG. 2. Act and Red production by *S. lividans* 66 (parental strain) and the *rif* mutants. Incubation was carried out in GYM liquid medium at 30°C for 6 days, and Act and Red levels were determined as described in Materials and Methods.

that produced high levels of Act in liquid culture showed a high level of Act production in solid culture (Fig. 3).

(ii) Red production *S. lividans* 66 produces a slight amount of the red antibiotic, Red. Like Act production, Red production was upregulated by the introduction of certain *rif* mutations, as represented by the mutants RC-1, EN-1, and DV-1 (Fig. 2 and 3 and Table 1). However, certain *rif* mutants (e.g., RC-1, CT-1, and SP-1) were shown to be able to upregulate Red only in certain growth media. Importantly, as examined with GYM liquid culture, the ability to upregulate Act correlated well with the ability to upregulate Red, except for the *rif-9* mutation (strain EN-1), which activated Red but not Act (Fig. 2)—although the correlation was less apparent when examined with R4 plate cultures (Fig. 3).

Effects of *rif* mutations on growth, sporulation, and CDA biosynthesis. We found that certain *rif* mutations markedly influence sporulation; this was especially pronounced in the white mutant EN-1, which lost almost completely the ability to sporulate on GYM plates, as determined by optical microscopy (Fig. 3).

To determine whether *rif* mutations influence production of CDA in *S. lividans*, three representative *rif* mutants (EN-1, SL-1, and RC-1) and the wild-type strain were monitored on R4 and SMMS agar plates. As shown in Fig. 4, all three mutants produced CDA on the SMMS plate. The productivity of CDA was especially pronounced when strain EN-1 was cultivated on the R4 plate. In contrast, strains RC-1 and SL-1 failed to produce CDA on the R4 plate, indicating medium dependence for CDA production. Thus, like Act and Red, production of CDA can be activated by certain *rif* mutations.

Expression of *act*II-ORF4, *act*IV-ORF1, *redZ*, and *redD*. (i) Western blotting analysis. The ActII-ORF4 protein, which is encoded by the *act*II-ORF4 gene, has been characterized as a DNA-binding protein that positively regulates the transcription of the Act biosynthesis genes in *S. coelicolor* A3 (2). One of the Act biosynthesis genes, *act*IV-ORF1, encodes a dehydrogenase that catalyzes an early reductive step in the Act biosynthesis pathway (17). Red biosynthesis in *S. coelicolor* is known to depend on two pathway-specific regulatory genes: *redD* and *redZ*. Moreover, *redD* transcription is highly dependent on *redZ*, and transcription of *redZ* appears to be negatively autoregulated (41, 43). We analyzed the expression patterns of these key genes, comparing the mutants with the wild-type strain. We used three mutants (SL-1, RC-1, and EN-1) as representative *rif* mutants, since these three produced an abundant amount of Act and/or Red (Fig. 2). The profile of Act and Red production in GYM medium is shown in Fig. 5A. Western analysis clearly demonstrated that ActII-ORF4 is upregulated in the mutants SL-1 and RC-1 (Fig. 5B), but not in the mutant EN-1 which did not produce Act (Fig. 5A). The biosynthesis gene product ActIV-ORF1 was expressed just after the upregulation of ActII-ORF4 occurred in mutants SL-1 and RC-1 (Fig. 5B), followed by the onset of Act production (Fig. 5A). It was surprising that, although the wild-type strain accumulated a considerable amount of ActII-ORF4, only a slight amount of ActIV-ORF1 was detected (Fig. 5B).

Results from Western analysis of RedZ and RedD also clearly indicate a relationship between the expression of these genes and Red production (Fig. 5C). All three mutants exhibited an upregulation of RedZ. It should be pointed out that RedD was expressed immediately after the upregulation of RedZ occurred (Fig. 5C), reflecting the onset of Red production (Fig. 5A). Thus, the *rif-14* and *rif-17* mutations (in strains SL-1 and RC-1) enable cells to activate two signal transduction pathways, the ActII-ORF4/ActIV-ORF1 and RedZ/RedD pathways, whereas the *rif-9* mutation (in strain EN-1) can activate only the RedZ/RedD pathway.

(ii) S1 nuclease protection assay. We conducted S1 nuclease protection assays to detect the level of transcripts of *act*II-ORF4, *redZ*, and *redD* (Fig. 6). The results obtained showed good correlation between the levels of transcripts of each gene (Fig. 6) and the actual amount of protein produced for ActII-ORF4, RedZ, and RedD (Fig. 5B and C), accounting for the elevated levels of these regulatory proteins in the *rif* mutants examined. It is also notable that the expression of *act*II-ORF4 and *redD* (and *redZ*) is characterized to be growth phase dependent, as has been shown in *S. coelicolor* A3(2) (17, 41). Importantly, unlike the wild-type strain, the mutant strains

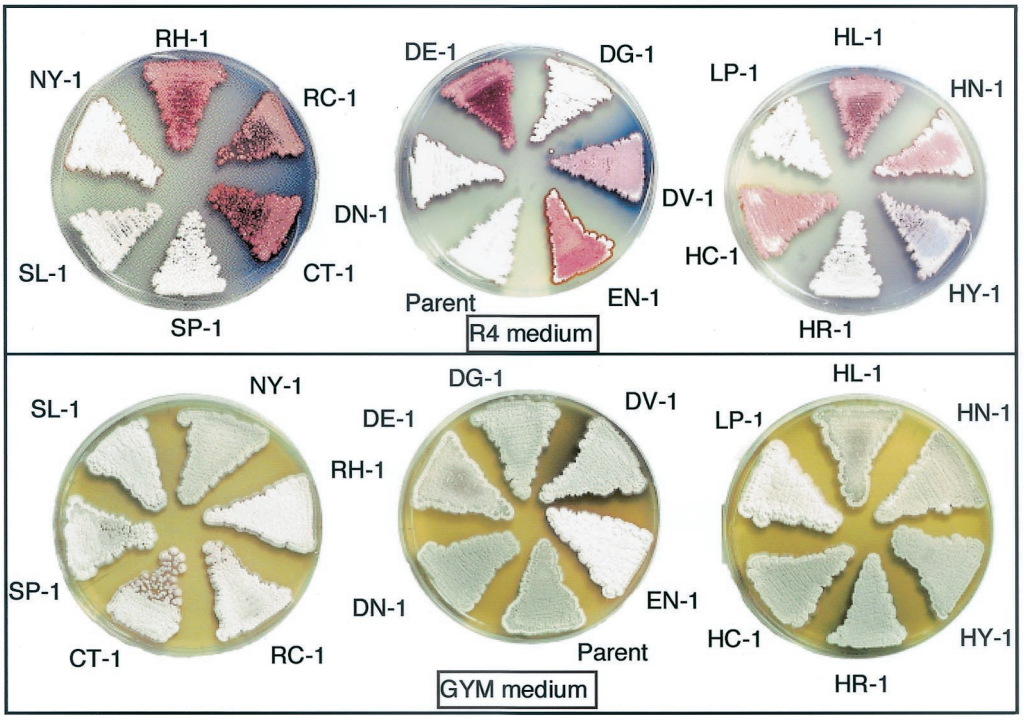

FIG. 3. Antibiotic production, aerial mycelia formation, and sporulation by *S. lividans* 66 and the *rif* mutants. Spores were inoculated on R4 or GYM agar plates and incubated at 30°C for 6 days. The blue color represents Act, and the red color represents Red.

EN-1 and SL-1 revealed growth-phase-independent expression of *redZ* and *act*II-ORF4, respectively (Fig. 6).

DISCUSSION

The results described here establish that the biosyntheses of Red and Act in *S. lividans* can be activated by introducing mutations (*rif*) that confer resistance to rifampin and that this activation is dependent on the mutation's position and the species of amino acid altered in the β-subunit of RNA polymerase. Moreover, specific *rif* mutations can activate CDA biosynthesis, suggesting that regulating antibiotic biosynthesis by *rif* mutation is pleiotropic. Consistent with previous studies (7, 41–43), the activation of Red and Act biosynthesis in *S. lividans rif* mutants results from the activated expression of *redD* and *act*II-ORF4, which are pathway-specific regulatory genes. Despite the lack of detail for the regulatory cascade of these two pathways, our finding may be helpful in the elucidation of the regulatory mechanisms for antibiotic biosynthesis in *Streptomyces*.

The taxonomy of *S. lividans* 66 is closely related to *S. coelicolor* A3(2) (25). Nevertheless, it is well known that although it possesses complete Act and Red biosynthesis gene clusters, *S. lividans* produces no or only a slight amount of Act and Red. What is the mechanism that represses Act and Red biosynthesis in *S. lividans*? Previous studies addressed this point and partly uncovered this mechanism, focusing on the *cutRS*, *afsR2*, and *orf10* genes of *S. lividans.* Although the *cutRS* signal transduction system and LysR-type transcriptional regulation mediated by *orf10* both function to repress the biosynthesis of Act (7, 30), *afsR2* positively regulates the biosynthesis of Act and

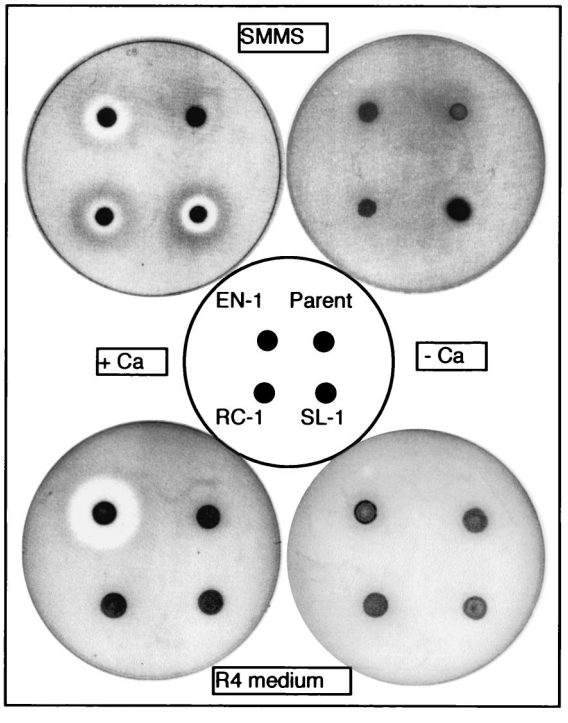

FIG. 4. Determination of CDA produced by *rif* mutants (EN-1, RC-1, and SL-1) of *S. lividans* 66. The strains were grown on SMMS and R4 agar plates at 30°C for 48 h. Details of the CDA assay conditions are described in Materials and Methods.

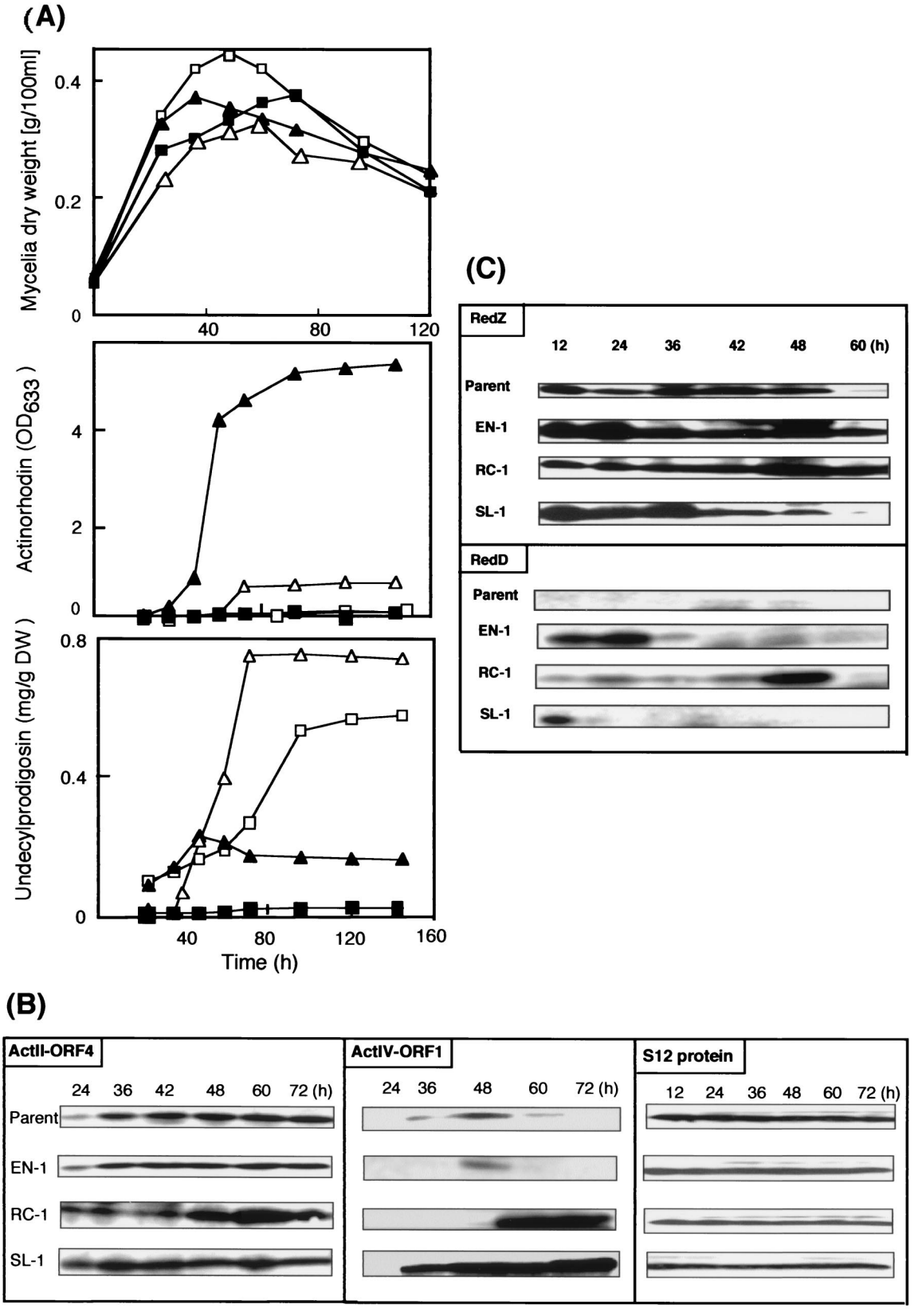

FIG. 5. Act and Red production and Western blotting analysis of the ActII-ORF4, ActIV-ORF1, RedZ, and RedD proteins. (A) Mycelial growth and antibiotic production by *S. lividans* 66 and the *rif* mutants in GYM liquid cultures. Symbols: ■, *S. lividans* 66 (wild-type strain); □, EN-1 (*rif-9*); ▲, SL-1 (*rif-14*); △, RC-1 (*rif-17*). (B) Western analysis of the ActII-ORF4, ActIV-ORF1, and ribosomal S12 proteins. Cells grown in GYM liquid cultures at 30°C were harvested at the indicated time, followed by the preparation of cell extracts for Western analysis. The expression of the ribosomal S12 protein was analyzed in parallel as an internal control. Each lane contained 20 μg of total proteins. (C) Western analysis of the RedZ and RedD proteins. Cells used for this analysis were same as those used in panel B.

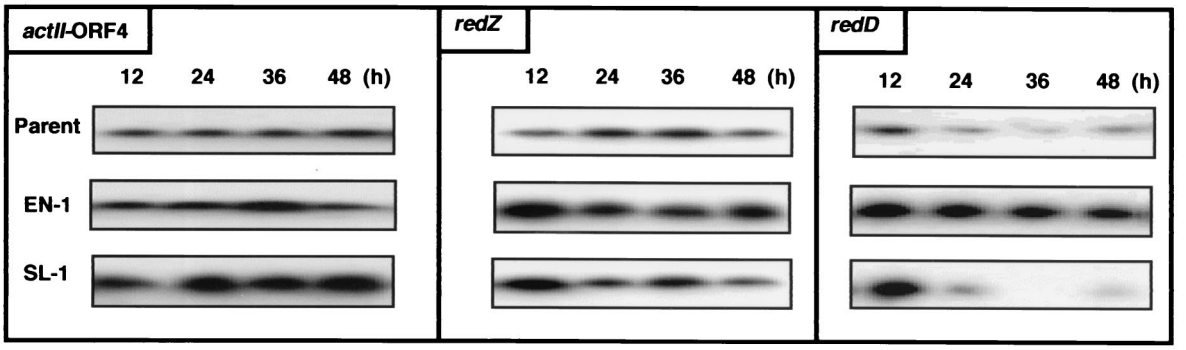

FIG. 6. Expression of *act*II-ORF4, *redZ*, and *redD* mRNA in parent (wild-type), EN-1 (*rif-9*), and SL-1 (*rif-14*) strains. RNAs for S1 nuclease protection assays were isolated from cells grown in the same GYM liquid medium for Western analysis. The probes for *act*II-ORF4, *redZ*, and *redD* were described in Materials and Methods.

Red (42). *afsR2*, when present at a high copy number, stimulates transcription of biosynthetic and regulatory genes in the Act gene cluster (*act*) and also stimulates the synthesis of Red (42). The *cutRS* operon is the second two-component system found in *Streptomyces* that negatively regulates antibiotic production; introduction of a mutation into *cutR* or *cutS* results in accerelated and increased production of Act (7). Likewise, disruption of the *orf10* gene, encoding the LysR-type transcriptional regulator, causes Act overproduction (30). In addition to these previous findings, our present observations suggest that the RNA polymerase may also be involved in the repression of Act and Red biosynthesis in *S. lividans*. Recent studies dealing with *S. coelicolor* suggest that both *redD* and *act*II-ORF4 genes are recognized in vivo by the RNA polymerase holoenzyme containing σ^{HrdB} (3, 16, 18). Consequently, it is possible that the *rif* mutations, which are able to activate Act and/or Red biosynthesis, alter the three-dimensional structure or the conformation of the β-subunit of RNA polymerase, thereby stabilizing the σ^{HrdB}-RNA polymerase complex and resulting in the efficient transcription of *act*II-ORF4 and *redZ* (and *redD*). The accelerated expression of *act*II-ORF4, *redZ*, and *redD* in mutants SL-1 and RC-1 (Fig. 5) supports this hypothesis. Also, it is likely that the dependence of activation on the mutation position in the *rpoB* gene and the resulting type of amino acid substitution can be attributed to forming different conformation and/or three-dimensional structures of the β-subunit of RNA polymerase. It is worth mentioning that, in contrast to the *rif-14* and *rif-17* mutations (in SL-1 and RC-1), the *rif-9* mutation (in EN-1) resulting in the deletion of Asn-430 failed to activate Act production, but *rif-9* was quite effective in activating Red production (Fig. 2). It is conceivable that the altered conformational status of the RNA polymerase resulting from *rif-9*, *rif-14*, or *rif-17* gave rise to different promoter selectivity (or affinity), leading to different expression levels of *act*II-ORF4 and *redZ*. Differential effects of *rif* mutations on the ability to sporulate (Fig. 3) may be explained in a similar way.

Previous studies of various bacteria, including *S. coelicolor*, demonstrated that mutations in the *rpoB* gene, which encodes the β-subunit of RNA polymerase, are responsible for the acquisition of resistance to rifampin (1, 36, 44). Almost all of the mutations are located in several conserved regions. In the present study, 17 kinds of mutations were detected in regions I, II, and III of the *rpoB* gene. Recent studies demonstrated that ppGpp binds to the β-subunit of *E. coli* RNA polymerase (11) and plays a causal role in activating *act*II-ORF4 transcription in *S. coelicolor* (18). These observations, taken together with our present and previously published observations (6, 33, 34, 40), show that the guanine nucleotide ppGpp is a pivotal signal molecule for initiating the onset of antibiotic production. ppGpp (and pppGpp) is believed to be responsible for the stringent response, which causes an immediate cessation of RNA synthesis and other cellular reactions (reviewed by Cashel et al. [5]). It is reasonable to consider that the enhanced expression of *redZ* (and *redD*) or *act*II-ORF4, which accompanies the activation of Red or Act biosynthesis, is based at least partly on the independence of cells from ppGpp for initiating the secondary metabolism. Thus, the mutated RNA polymerase may function by mimicking the ppGpp-bound form.

In *S. coelicolor*, transcription of the activator genes *act*II-ORF4 and *redD* has been characterized to be growth phase dependent, and the transcription of the antibiotic structural genes *act*IV-ORF1 (for Act) and *redP* (for Red) has been shown to occur after the accumulation of almost maximal levels of *act*II-ORF4 or *redD* transcripts (17, 41). This suggests that a threshold concentration of the activator is needed for transcription of the antibiotic genes. Consistent with this, our present work with *S. lividans* also demonstrated a growth phase dependence of the transcription of *act*II-ORF4, *redZ*, and *redD* in the wild-type strain (Fig. 6). However, the low level of ActII-ORF4 or RedZ protein expressed in wild-type *S. lividans* 66 (Fig. 5B and C) apparently was not enough to activate Act or Red biosynthesis. In contrast, in *rif* mutants expression of the *act*II-ORF4 or *redZ* was high during the transition or stationary phase, or even during the early growth phase (for *redZ*), resulting in the activation of Act or Red biosynthesis (Fig. 5 and 6).

It has been demonstrated that cluster I (region II in our study), which harbors most of the *rif* mutations, is the part of the "5′-face" of the active center and is the rifampin-binding site (37). Moreover, cluster I (which encodes amino acids 400 to 466) interacts with the +1 position of DNA-RNA hybrid in the transcription elongation complex (28). Consequently, it is possible that *rif* mutations in region II of the *rpoB* gene activate Act or Red biosynthesis by the efficient transcription of the activator genes such as *act*II-ORF4, *redZ*, and *redD* (or the inefficient transcription of the repressor genes). The dependence of activation on the mutation position and type of amino acid substitution implicates the existence of a relationship be-

tween the function and structure of the β-subunit of RNA polymerase in initiating bacterial secondary metabolism.

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