Activation of Dormant Bacterial Genes by Nonomuraea sp. Strain ATCC 39727 Mutant-Type RNA Polymerase

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Actinomycetes are ecologically important microorganisms relevant for their cell cycle and ability to produce over 70% of naturally occurring antibiotics (6). However, recent advances in genome research have revealed that the ability of actinomycetes to produce antibiotics and other bioactive secondary metabolites has been underestimated due to the presence of cryptic gene clusters. The activation of dormant genes is therefore one of the most important areas of experimental research for the discovery of drugs in these organisms. There is accumulating evidence that the ability of actinomycetes to produce antibiotics and other bioactive secondary metabolites has been underestimated due to the presence of cryptic gene clusters (2, 27, 28, 40). The activation of dormant genes encoding bioactive molecules is therefore one of the most important areas of experimental research for the discovery of new drugs in these organisms.

There have been many studies of the model organism Streptomyces coelicolor A3(2), which produces at least four antibiotics: the pigmented actinorhodin (Act) and undecylprodigiosin (Red), methylenomycin, and calcium-dependent antibiotic (CDA). Streptomyces lividans, a fast-growing close relative of S. coelicolor A3(2), has a number of advantages for use as a model system in which to investigate the activation of dormant antibiotic gene expression, including its production of little or no Act and Red despite the existence of all the required biosynthetic genes (11, 18). GDP 3'-diphosphate (ppGpp), which accumulates upon nutritional downshift, is a global regulator of gene expression, elicits secondary metabolism in bacteria (5, 9, 10, 17, 22, 23, 25, 30, 34, 38), and is thus called a bacterial alarmone (1). ppGpp binds RNA polymerase (RNAP) and causes a redirection of transcription so that genes important for starvation survival and virulence are favored at the expense of those required for growth and proliferation (the so-called stringent response) (4). Interestingly, certain rifampin resistance (rif) mutations, mapped within the RNAP β-chain-encoding gene rpoB, result in ppGpp-independent antibiotic production in S. coelicolor A3(2) and activate Act, Red, and CDA biosynthesis in S. lividans, suggesting that they can functionally mimic the modification induced by the binding of ppGpp (11, 39). The efficacy of RNAP mutations in activating the production of a dormant antibiotic (3,3'-neotrehalosadiamine) in Bacillus subtilis has also been shown previously (12).

The recent observation that several actinomycetes possess two rpoB genes, in contrast to the widely accepted consensus of the existence of a single RNAP in bacteria, has suggested the possibility of developing new strategies to activate dormant gene expression in bacteria. rpoB paralogs in Nonomuraea sp. strain ATCC 39727 (previously called Actinomadura), which produces the glycopeptide antibiotic A40926, were the first to be reported (37), and rpoB paralogs in Nocardia farcinica, an emerging pathogen in immunocompromised patients, were then observed (13). In Nonomuraea sp. strain ATCC 39727, rpoB(S) and rpoB(R) provide the microorganism with two functionally distinct and developmentally regulated RNAPs. Major differences between the rpoB(S) and rpoB(R) paralogs are depicted in Fig. 1A. rpoB(R) is characterized by an 18-bp in-frame deletion and mutations causing five amino acid substitutions (H426N, S431N, F445M, S474Y, and M581D), located within or close to the so-called rif clusters that play a key role in fundamental activities of RNA polymerase. Here, we report that rpoB(R) markedly activated antibiotic biosynthesis in the wild-type Streptomyces coelicolor strain 1326 and also in strain KO-421, a relaxed (rel) mutant unable to produce ppGpp. Site-directed mutagenesis demonstrated that the rpoB(R)-specific missense H426N mutation was essential for the activation of secondary metabolism. Our observations also indicated that mutant-type or duplicated, rpoB often exists in nature among rare actinomycetes and will thus provide a basis for further basic and applied research.
role in fundamental activities of RNAP (39). Of these substitutions, H426N was of particular interest because it corresponds to one of the rifampin resistance rpoB mutations that activate antibiotic production in S. lividans, H437N (11). Indeed, enhanced expression of rpoB(R) in Nonomuraea sp. strain ATCC 39727 causes overproduction of the glycopeptide A40926 in this organism (37). This observation prompted us to investigate the ability of Nonomuraea rpoB(R) to activate antibiotic production in Streptomyces spp., which have the potential to produce a wide variety of antibiotics. Here, we report the efficacy of rpoB(R) as a tool for the activation of cryptic antibiotic biosynthesis in S. lividans. The physiological significance of rpoB duplication is also discussed based on the results from actinomycetes isolated from soil.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. Nonomuraea sp. strain ATCC 39727 was obtained from the American Type Culture Collection (ATCC).
This strain was deposited under the genus name Actinomadura and later classified as Nonomuraea. Yeast-starch medium was used for growth, and culture conditions were as reported previously (36). *S. lividans* strains 1326 (wild type), KO-421 (relC), and KO-422 (relC rif1) used in this study were described previously (18, 20). These strains were grown on R3 or R4 medium (32) at 30°C. R4 medium was the same as R3 medium but with a reduced amount of yeast extract (0.1% instead of 0.5%) and no added KH2PO4. Culture conditions for *S. lividans* were as reported previously for *S. coelicolor* A3(2) (10).

**Plasmids and DNA procedures.** The *rpoB(R)* gene, including its own promoter region, was cloned into the BamHI site of pUC18 from a genomic library of *Nonomuraea* sp. strain ATCC 3972, which was constructed by partial restriction digestion of the bacterial DNA with BglII (Fig. 1A). The *rpoB(R)* gene was then excised as an EcoRI-XbaI fragment 4,044 bp in length. This fragment was ligated into pTYM18 (29), resulting in pTYM-rpoB(R), carrying *rpoB(R)* in the same orientation as lacZ (Fig. 1B). Plasmids were introduced into the *S. lividans* strains by conjugation with *Escherichia coli* GM2929pUB307::Tn7 as described previously (18). To allow plasmid selection, conjugation medium was supplemented with kanamycin (25 μg/ml). Site-directed mutagenesis of *rpoB(R)* was performed using a QuickChange II site-directed mutagenesis kit in accordance with the instructions of the manufacturer (Stratagene, La Jolla, CA). The primers used for mutagenesis were N426H-F, N426H-R, N426Y-F, N426Y-R, N426F-R, and N426R-R (Table 1). The amplification reaction consisted of 35 cycles including denaturation at 94°C for 1 min, and an extension step at 72°C for 1 min. Amplification reactions were performed using the 5′-end-labeled primer PEX2 and SuperScript reverse transcriptase (Gibco-BRL, Grand Island, NY) according to standard procedures (31).

**Antibiotic assays.** Act and Red production was determined as described previously (11). CDA was assayed according to the method of Kiers et al. (18) by using *Micrococcus luteus* as a test organism. Rifampicin SV was evaluated by extraction with ethyl acetate, followed by high-performance liquid chromatography (HPLC) analysis using an octadecylsilane 5C18-MS-II column (4.6 by 250 mm; Nacalai Tesque, Kyoto, Japan).

**Isolation and identification of rifampin-resistant strains from soil.** Nature samples isolated from various locations in Tsukuba, Japan, were suspended in sterilized water, and aliquots of the supernatant were plated onto glucose-yeast extract-malt extract (GYM) plates (23) containing 30 μg/ml of rifampin and 10 μg/ml of amphotericin B to suppress the growth of fungi. After a 10-day incubation at 30°C, the colonies that developed on the plates were purified by single-colony isolation and used for further analyses. The identification of each strain was performed by sequencing 16S RNA with primers 16S-9F (GAGTTTGATCCTGGCTCA) and ActinoRpoA-R3 (Table 1). The strains were classified as belonging to the species with the highest degree of sequence similarity (>98% in general). The *rpoB* genes of actinomycetes were sequenced using primers RpoB-F and RpoB-R or ActinoRpoF1 and ActinoRpoB-R, while *poB* genes were sequenced using ActinoRpoC-F1 and ActinoRpoC-R1 or ActinoRpoC-F2 and ActinoRpoC-R2. The *poB* genes were sequenced using ActinoRpoF-A and ActinoRpoC-R-A or ActinoRpoF-R and ActinoRpoC-R-R (Table 1).

Analyses of the growth of and Act and Red production by *S. lividans* 1326, KO-421 (relC), KO-422 (relC rif1), and transconjugants harboring *rpoB(R)*, mutated *rpoB(R)* (expressing N426Y, N426H, or N426H), or control empty vector pTYM18 in R3 or R4 liquid medium are shown in Fig. S1 in the supplemental material. The results of the microbiological assay for CDA production by *S. lividans* 1326, KO-421 (relC), KO-422 (relC rif1), and transconjugants harboring *rpoB(R)* or control empty vector pTYM18 after 96 h of growth on R3 or R4 agar medium are shown in Fig. S2 in the supplement material. For the morphological appearance of “Nonomuraea territana” strains, S114 and S88 during growth on GYM agar medium at 30°C for 3 and 10 days, see Fig. S3 in the supplemental material.

**RESULTS**

**Expression of Nonomuraea *rpoB(R)* in *S. lividans.* To assess the efficacy of the ectopic expression of *Nonomuraea rpoB(R)* in the activation of secondary metabolism, *S. lividans* was used as a model organism. The *rpoB(R)* gene, including its putative promoter region, was cloned into the *E. coli*-Streptomyces conjugative vector pTYM18 (Fig. 1B) and introduced into *S. lividans* 1326. The presence of *rpoB(R)* in *S. lividans* transconjugants was confirmed by PCR analysis using the primer pair Δ18-1/Δ18-2 (Table 1) spanning the *rpoB(R)*-specific 18-bp deletion (Fig. 1C). Transconjugants thus obtained were all resistant to high levels of rifampin (up to 200 μg/ml) (Fig. 1D), suggesting that *rpoB(R)* was expressed in the host. The expression of *rpoB(R)* was further confirmed by the results of primer extension experiments. In *Nonomuraea*, two major developmentally regulated transcriptional start sites, TSS1 and TSS2 (Fig. 2A, lanes 3 to 6), are located 43 and 126 nt upstream of the putative translational start codon, respectively (Fig. 2B). The levels of transcripts starting at TSS2 increased markedly during late growth (168 h) (Fig. 2A, lanes 5 to 6), thereby reinforcing *rpoB(R)* expression during the stationary phase.
consistent with the results reported previously (37). In contrast to this observation, the transcription of rpoB in S. lividans transconjugants did not start at TSS2 but was strongly promoted by TSS1 (Fig. 2A, lanes 1 and 2).

Activation of Act, Red, and CDA biosynthesis by Nonomuraea rpoB(R).

The growth of antibiotic production by S. lividans 1326 and its transconjugants on R3 and R4 agar media were analyzed. R4 medium was characterized by a lower content of yeast extract than that in R3 medium and no added phosphate, potentially resulting in phosphate-limited growth. Under these conditions, activation of the biosynthesis of Red (mycelium-associated red pigment) and Act (diffusible blue pigment) in S. coelicolor A3(2) is not subject to stringent control (5, 33). Our results indicated that S. lividans transconjugants produced markedly larger amounts of Red (Fig. 3A), Act (Fig. 3B), and CDA (Fig. 3C) than the wild type on both R3 and R4 agar media.

To better quantify the extent of this phenomenon, antibiotic production in R3 and R4 liquid media was evaluated (Fig. 4; also see Fig. S1 in the supplemental material). Analyses of the growth curves confirmed the absence of any significant effect of the presence of rpoB(R) on the growth rate (see Fig. S1 in the supplemental material). In contrast, Red and Act biosynthesis levels were markedly stimulated by the transgene. The maximal increase of Act in R4 medium was 25-fold or more (Fig. 4). It is notable that Red and Act biosynthesis in rpoB(R) transconjugants started earlier than that in the wild-type strain (i.e., production was precocious) and occurred largely during the exponential growth phase (see Fig. S1 in the supplemental material).

Mechanism of activation of antibiotic biosynthesis by Nonomuraea rpoB(R).

To confirm that the observed activation of secondary metabolism was due specifically to the expression of rpoB(R) rather than to an imbalance in RNAP assembly as a consequence of an excess of β-chains, we mutagenized the rpoB(R) asparagine codon at position 426, reverting asparagine back to the common amino acid histidine (N426H). In addition, the same codon was changed to express tyrosine (N426Y) and arginine (N426R) as positive controls because there is evidence that these two amino acid substitutions, when introduced into the S. lividans RNAP β-chain, activate antibiotic production (11, 20). The mutagenized genes were cloned into pTYM18 and then transferred into S. lividans 1326 as described above. rpoB(R) with the N426H reversion did not show stimulation, but instead showed a reduction of antibiotic production under all conditions examined (Fig. 3 and 4). In contrast, rpoB(R) forms harboring the N426Y and N426R missense mutations were as effective as the wild-type rpoB(R), as expected.

To gain insight into the molecular mechanisms underlying the stimulation of secondary metabolism, we examined the ability of rpoB(R) to reactivate antibiotic biosynthesis in S. lividans KO-421, a relaxed (relC) mutant unable to produce antibiotic due to the lack of ppGpp synthesis. The effect of
rpoB(R) on antibiotic production was also tested with *S. lividans* KO-422, a double-mutant (relC rif1) derivative of KO-421. In this strain, a rif mutation, H437Y (rif1), affecting the histidine residue corresponding to the residue that is changed to asparagine in rpoB(R) (H426N), partially restored the antibiotic-producing phenotype (11, 20). We detected the reactivation of Act, Red, and CDA production by rpoB(R) to wild-type levels in relC transconjugants grown in R3 medium.
TABLE 2. List of actinomycetes with mutant-type rpoB or duplicated rpoB isolated from soila

<table>
<thead>
<tr>
<th>Strain</th>
<th>Species classificationb</th>
<th>Level of resistance to rifampin (smallest antimicrobiogram affecting growth)c</th>
<th>Antibiotic productivityd</th>
<th>Polymorphism of rpoBe</th>
<th>Alteration(s) detected in amino acid sequence corresponding to rif clusterf</th>
</tr>
</thead>
<tbody>
<tr>
<td>S14</td>
<td>Amycolatopsis kentuckyensis</td>
<td>300</td>
<td>+</td>
<td>Single</td>
<td>None (reference sequence)</td>
</tr>
<tr>
<td>S15</td>
<td>Amycolatopsis mediterranei</td>
<td>200</td>
<td>+f</td>
<td>Single</td>
<td>D427Q, L432L, S433D</td>
</tr>
<tr>
<td>S18</td>
<td>Streptomyces spectabilis</td>
<td>200</td>
<td>+</td>
<td>Single</td>
<td>A443S</td>
</tr>
<tr>
<td>S24</td>
<td>Streptomyces albogriseolus</td>
<td>&gt;300</td>
<td>+f</td>
<td>Single</td>
<td>D427Q, L432L, S433D</td>
</tr>
<tr>
<td>S36</td>
<td>Streptomyces violaceus</td>
<td>&gt;300</td>
<td>+</td>
<td>Single</td>
<td>H437Y</td>
</tr>
<tr>
<td>S57</td>
<td>Kribbella aluminosa</td>
<td>300</td>
<td>-</td>
<td>Single</td>
<td>I413L, L432V</td>
</tr>
<tr>
<td>S65</td>
<td>“Nonomuraea rubescens”</td>
<td>200</td>
<td>+</td>
<td>Single</td>
<td>Q425H</td>
</tr>
<tr>
<td>S108</td>
<td>Nocardia asiatica</td>
<td>300</td>
<td>+</td>
<td>Single</td>
<td>L422 M, Q424V, M426L, N429H, H437Q</td>
</tr>
<tr>
<td>S114</td>
<td>“Nonomuraea territana”</td>
<td>200</td>
<td>+</td>
<td>Single</td>
<td>I413 M</td>
</tr>
<tr>
<td>S58</td>
<td>“Nonomuraea territana”</td>
<td>200</td>
<td>+ Duplicate</td>
<td>I413 M, S433A, N442S</td>
<td></td>
</tr>
<tr>
<td>S66</td>
<td>“Actinomadura maheshkhiensis”</td>
<td>300</td>
<td>+ Duplicate</td>
<td>R440S, N442S</td>
<td></td>
</tr>
<tr>
<td>S79</td>
<td>Sphaerisporangium viridialbum</td>
<td>200</td>
<td>+ Duplicate</td>
<td>N429I, H437Q, N442S</td>
<td></td>
</tr>
<tr>
<td>S86</td>
<td>Nonomuraea helvata</td>
<td>200</td>
<td>+ Duplicate</td>
<td>I413 M, S433A, N442S</td>
<td></td>
</tr>
<tr>
<td>S94</td>
<td>Nocardia nitagatensis</td>
<td>200</td>
<td>+ Duplicate</td>
<td>N429I, G435A</td>
<td></td>
</tr>
</tbody>
</table>

a The rpoB sequence of each strain was compared with that of S. coelicolor A3(2). None of the mutations S412A, Q428E, N429T, S433A, G434S, and N442S in single rpoB were taken to indicate a mutant type because 412A, 428E, 429T, 433A, 434S, and 442S are wild type in either Bacillus subtilis, E. coli, Nonomuraea sp. strain ATCC 39727, Nocardia farcinica, Mycobacterium tuberculosis, or Thermus thermophilus.
b Assigned on the basis of 16S rRNA gene sequence similarity.
c Determined after 7 days of incubation on GYM agar.
d Agar plugs (diameter, 11 mm) were cut from each of the GYM, 2× GYM, and 1/2× GYM agar plates after 2 to 10 days of incubation at 30°C and placed onto the assay plate inoculated with Staphylococcus aureus 209P as a test organism.
e Numbering originated from the position corresponding to the start codon of rpoB. Boldface characters show mutations which were reported previously to cause rifampin resistance in S. lividans or S. coelicolor (unpublished data) (11, 39).
f The antibiotic produced was determined to be rifamycin SV.

Likewise, we detected remarkable stimulation of antibiotic production in relC rpoB(R) transconjugants grown in R4 medium, in which antibiotic biosynthesis was expected to be largely independent of stringent control (Fig. 4). These results suggest the existence of an additional mechanism(s) underlying rpoB(R) stimulation, as supported by the fact that the effects of rpoB(R) on the reactivation of secondary metabolism were additive to those of rif1 (i.e., H437Y), a mutation that was suggested to mimic ppGpp binding (Fig. 4; also see Fig. S1 and S2 in the supplemental material).

Distribution of bacteria with mutant-type rpoB or duplicated rpoB in nature. Next, we examined the frequency of the rpoB polymorphism in nature. We found that a number of colonies developed on plates containing a high concentration (30 μg/ml) of rifampin when soil samples were plated and incubated for 10 days. The frequency of rifampin-resistant isolates among total CFU was ~0.1%, as determined by comparing the CFU developed on GYM plates with and without rifampin. On the basis of the 16S rRNA gene sequencing, the majority (82%) of these rifampin-resistant isolates were classified as actinomycetes, including members of the genera Streptomyces (34 species), Nocardia (9 species), Nonomuraea (9 species), Mycobacterium (4 species), Amycolatopsis (3 species), Isoptericola (2 species), Actinomadura (1 species), Kribbella (1 species), Sphaerisporangium (1 species), and Sphaerisporangium (1 species). Other bacteria were classified as members of the genera Pseudomonas (3 species), Mitsuaria (3 species), Acidovorax (2 species), Comamonas (2 species), Stenotrophomonas (2 species), and six other genera.

All actinomycetes isolated here (75 strains) were subjected to rpoB sequencing focusing on rif clusters. As expected, several strains (S14, S15, S18, S24, S36, S57, S65, S108, and S114) were found to have mutant-type rpoB (Table 2). These Rif’ rpoB paralogs revealed high levels of similarity (80.4% in Nocardia asiatica S108 to 98.3% in Streptomyces violaceus S36) to the endogenous Rif’ rpoB gene in S. lividans. The remaining rifampin-resistant actinomycetes had no changes in their rif clusters. The mutations N442Y (in S24), H437Y (in S36), and H437Q (in S108) were especially notable, as these mutations were reported previously to cause rifampin resistance in S. lividans or S. coelicolor (11, 39). Strains S14, S15, and S108 were characterized by mutations at multiple amino acid positions. It is also notable that S14 and S15 were classified as species known to be rifamycin producers and that these two strains actually produced rifamycin SV, as determined by HPLC analysis (data not shown). In addition, five strains (S58, S66, S79, S86, and S94) classified as belonging to Nonomuraea, Nocardia, Actinomadura, or Sphaerisporangium showed polymorphism of the rpoB gene, which consisted of wild-type rpoB and mutant-type rpoB (Table 2). Polymorphism of rpoB genes was also analyzed by Southern hybridization. Preliminarily, we tested a number of restriction endonucleases (BamHI, BglIII, HinfI, KpnI, SacI, SalI, and PstI), selecting those that were able to cleave the DNA from all strains and demonstrated polymorphic cleavage sites within rpoB on the basis of the Southern hybridization data. In the analysis presented in Fig. 5, total DNA from the actinomycete strains was digested with either SacI or HinfI and probed with either a 565-bp (probe A) (Fig. 5B) or an 856-bp (probe B) (Fig. 5C) rpoB-specific DNA fragment. As the two probes were contiguous (although with partial overlap) on the physical map of the rpoB gene (Fig. 5A), the detection of two (or more) identical bands with both probes was indicative of the occurrence of two (or more) al-

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This analysis confirmed the \textit{rpoB} duplication in S58, S66, S79, S86, and S94. On the other hand, no duplication of \textit{rpoA} (coding for the RNAP \(\alpha\)-subunit) and \textit{rpoC} (coding for the RNAP \(\beta\)-subunit) was found, as determined preliminarily by the sequencing of these genes. Thus, \textit{rpoB} mutant forms are widely distributed among actinomycetes, while the mutant-type \textit{rpoB} with polymorphism appears to be distributed preferentially in the so-called rare actinomycetes. It is also notable that all rifampin-resistant isolates thus obtained (with the exception of \textit{Kribbella aluminosa}) were able to produce antibiotics (Table 2).

Possible significance of duplicated \textit{rpoB} gene in developmental strategy. As the strains S114 (with single \textit{rpoB}) and S58 (with duplicated \textit{rpoB}) revealed a high degree of similarity (98.8\%) to one another in the 16S rRNA gene sequence and were both classified as “\textit{Nonomuraea terrinata}” (Table 2; see Fig. S3 in the supplemental material for the morphological appearance), we examined the possible role of the duplicated \textit{rpoB} gene in developmental strategy by cultivating these two strains under a wide variety of growth conditions. As expected, strain S58 with duplicated \textit{rpoB} showed much greater capability than strain S114 for growth (representing primary metabolism) and aerial mycelium formation and antibiotic production (representing the developmental strategy) (Table 3), thus suggesting the physiological significance of \textit{rpoB} duplication.

**DISCUSSION**

Antibiotic biosynthesis in streptomycetes is a developmentally regulated process, with production in liquid medium associated with the stationary phase (3, 7, 24). The results of the present study demonstrated that \textit{rpoB(R)} expression in \textit{S. lividans} allows bacteria to bypass these regulatory circuits, resulting in the constitutive activation of the antibiotic biosynthetic pathways. Thus, in \textit{rpoB(R)} transconjugants, high levels of Act

**FIG. 5.** Detection of duplicated \textit{rpoB} genes by Southern blot hybridization. (A) Physical map of the \textit{rpoB(S)} and \textit{rpoB(R)} genes of \textit{Nonomuraea} sp. strain ATCC 39727 with the location of the probes A and B used in Southern blot hybridization. The relative positions of the following restriction sites are indicated above the genetic map: HinfI (H) and SacI (S). (B and C) Total DNA samples extracted from \textit{S. coelicolor} A3(2) 1147 (lanes 1), \textit{Amycolatopsis mediterranei} S15 (lanes 2), \textit{Streptomyces albogriseolus} S24 (lanes 3), \textit{Nocardia niigatensis} S94 (lanes 4), \textit{Nonomuraea} sp. strain ATCC 39727 (lanes 5), \textit{Nonomuraea helvata} S86 (lanes 6), “\textit{Actinomadura maheshkhaliensis}” S66 (lanes 7), \textit{Sphaerisporangium viridialbum} S79 (lanes 8), \textit{Nonomuraea} sp. strain ATCC 39727 (lanes 9), \textit{Nocardia asiatica} S108 (lanes 10), “\textit{Nonomuraea terrinata}” S114 (lanes 11), and “\textit{Nonomuraea terrinata}” S58 (lanes 12) were digested with either SacI or HinfI, as indicated, and probed with either probe A (panel B) or probe B (panel C). Positions of molecular size standards, in base pairs, are indicated to the left of each panel.
and Red production during the entire growth phase in either phosphate-limited or nonlimited medium could be detected (Fig. 4; also see Fig. S1 in the supplemental material). This finding indicates that rpoB(R) expression does not simply alleviate the stringent control of antibiotic biosynthesis, as initially suggested by the presence of the H426N missense mutation. Indeed, when the same H426N missense mutation was introduced into the *S. lividans* RNAP β-chain, the stimulatory effect on secondary metabolism was much less pronounced than that observed with the rpoB(R) transgene (11). Nevertheless, the results of the experiments with asparagine substitution at position 426 clearly demonstrated that H426N is crucial for *rpoB(R)-induced phenotype* (Fig. 3 and 4). It is therefore highly likely that the additional rif cluster-associated rpoB(R)-specific missense mutations (Fig. 1A) interact functionally with the H426N missense mutation, leading to the marked effect of

![Table 3](image-url)

**Table 3. Growth of and aeralmycelium formation and antibiotic production by Nonomuraea terrinata strains S14 and S58 under various culture conditions.**

<table>
<thead>
<tr>
<th>Cultivation temp (°C)</th>
<th>Medium&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Growth&lt;sup&gt;b&lt;/sup&gt; of:</th>
<th>Aeralmycelium formation&lt;sup&gt;b&lt;/sup&gt; by:</th>
<th>Antibiotic production&lt;sup&gt;b&lt;/sup&gt; by:</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>S14</td>
<td>S58</td>
<td>S14</td>
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<tr>
<td>30</td>
<td>GYM</td>
<td>+++</td>
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<td>+++</td>
</tr>
<tr>
<td></td>
<td>GYM (pH 5)</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>GYM (pH 9.5)</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>20× GYM</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>1/2× GYM</td>
<td>++</td>
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<td>GYM–1% yeast extract</td>
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<td>GYM–1% yeast extract</td>
<td>++</td>
<td>++</td>
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</tr>
<tr>
<td>GYM–1% malt extract</td>
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<td>GYM–2% malt extract</td>
<td>++</td>
<td>++</td>
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<tr>
<td>LB</td>
<td>++</td>
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<td>1/2× LB</td>
<td>++</td>
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<tr>
<td>SYM</td>
<td>++</td>
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<tr>
<td>1/2× SYM</td>
<td>++</td>
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<td>++</td>
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<tr>
<td>2× SYM</td>
<td>++</td>
<td>++</td>
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</tbody>
</table>

<sup>a</sup> Cultivation was performed using agar plates for 15 days at 30, 43, or 20°C. Luria-Bertani (LB) medium contained 1% tryptone (Difco), 0.5% yeast extract (Difco), and 0.5% NaCl. SYM medium contained 1% soluble starch instead of the glucose in GYM medium.

<sup>b</sup> –, no growth or no aeralmycelium formation; +, sparse; ++, moderate; ++++, abundant; NA, not applicable due to lack of growth.

<sup>c</sup> Agar plugs (diameter, 11 mm) were cut from the cultivation plate every 24 h and put onto the agar plate inoculated with *Staphylococcus aureus* 209P as a test organism, and the plate was then incubated for 16 h at 37°C. –, no inhibition zone; +, inhibition zone diameter of ≤15 mm; ++, diameter of 16 to 19 mm; ++++, diameter of ≥24 mm.
rpOB (R). From a practical viewpoint, our findings suggest the intriguing possibility of using rpOB(R)-based technology to improve strains and to search for novel bioactive molecules by activating dormant genes. This technology should have greater potential than simple rif selection currently used to improve the production of secondary metabolites (10, 26, 35), as the introduction of rpOB (R) was eightfold more effective for antibiotic production in R4 medium than the introduction of simply the H426Y mutation (i.e., the rif1 mutation) (Fig. 4).

The clusters of mutations in rpOB that confer rifampicin resistance were initially discovered in E. coli by Jin and Gross (14, 16). While our results seem to have immediate industrial applications, the molecular mechanisms responsible for the observed phenomena are not completely understood. Most rpOB (R)-specific missense mutations affect residues evolutionarily conserved in all prokaryotes and are predicted to modify the structure-function relationships of the RNAP β-chain subunit. Thus, rif cluster-associated mutations frequently alter promoter clearance, transcription elongation, and transcription termination (15, 16, 19, 21). It is worth noting the presence of both H426N (rif cluster I) and S474Y (rif cluster II) missense mutations in the corresponding regions of rpOB2 from Nocardia farcinica, an actinomycete that shares with Nonomuraea sp. strain ATCC 39727 the distinction of having two rpOB paralogs (rpOB and rpOB2) (13).

Another interesting point is the physiological significance of rpOB duplication in these prokaryotes. In Nocardia farcinica, as well as in other Nocardia species, rpOB duplication has been shown previously to contribute to rifampicin resistance. As certain rif mutations in Mycobacterium tuberculosis have been shown to reduce the growth rate, it has been suggested that such disadvantages can be minimized by carrying both the wild-type rpOB and a mutant rpOB [rpOB (R) or rpOB2] (13). However, the more intriguing possibility is that rpOB duplication may contribute to the developmental strategy of several actinomycetes. This hypothesis is supported by the observation that rpOB (R) transcription is tightly regulated during Nonomuraea growth (Fig. 2) and that the Constitutive expression of this gene increases the production of the glycopeptide antibiotic A40926 in this organism (37). The greater capabilities for aerial mycelium formation (and thus sporulation) and antibiotic production observed in “Nonomuraea terrinata” strain SS8 with a duplicated rpOB gene (Table 3) also appear to support this hypothesis.

It was rather surprising that rpOB gene polymorphism was detected frequently among rifampicin-resistant actinomycetes isolated from nature (5 of 75 strains), although it was preferentially distributed in rare actinomycetes (not Streptomyces spp.) (Table 2). This finding suggests that these rare actinomycetes have utilized this polymorphism to survive in nature by elegantly adjusting the gene expression as discussed above. From this viewpoint, it is notable that rifamycin producer strains S14 and S15 had a rif cluster sequence distinguished from those of other actinomycetes (Table 2). It is also intriguing to examine whether various rpOB (R) forms found in nature (including the five rpOB (R) forms found in this study) exhibit greater capabilities to activate the bacterial dormant genes than the Nonomuraea sp. strain ATCC 39727 rpOB (R). Thus, understanding the natural rpOB status and its utilization for dormant gene activation appears to provide new horizons for medical and industrial microbiology.

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REFERENCES


Practical Streptomyces genetics. The John Innes Foundation, Norwich, United Kingdom.


