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Activation and Products of the Cryptic Secondary Metabolite Biosynthetic Gene Clusters by Rifampin Resistance (*rpoB*) Mutations in Actinomycetes

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A subset of rifampin resistance (*rpoB*) mutations result in the overproduction of antibiotics in various actinomycetes, including *Streptomyces*, *Saccharopolyspora*, and *Amycolatopsis*, with H437Y and H437R *rpoB* mutations effective most frequently. Moreover, the *rpoB* mutations markedly activate (up to 70-fold at the transcriptional level) the cryptic/silent secondary metabolite biosynthetic gene clusters of these actinomycetes, which are not activated under general stressful conditions, with the exception of treatment with rare earth elements. Analysis of the metabolite profile demonstrated that the *rpoB* mutants produced many metabolites, which were not detected in the wild-type strains. This approach utilizing rifampin resistance mutations is characterized by its feasibility and potential scalability to high-throughput studies and would be useful to activate and to enhance the yields of metabolites for discovery and biochemical characterization.

Actinomycetes produce a variety of natural products that are of major importance in the pharmaceutical industry. More than 50% of all anti-infective and anticancer compounds developed over the past 25 years have been natural products or derivatives thereof (1). Discovery of novel antibiotics and strain improvement for overproduction are important in applied microbiology research, especially in the production of clinically important antibiotics as well as antibiotics important in veterinary medicine and agriculture. 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. That is, genome sequencing projects have revealed many biosynthetic gene clusters for the production of unknown secondary metabolites. For example, *Streptomyces coelicolor*, *Streptomyces avermitilis*, *Streptomyces griseus*, and *Saccharopolyspora erythraea* are each known to produce three to five secondary metabolites but actually possess >20 clusters that encode known or predicted biosynthetic pathways for secondary metabolites (2–5). Exploitation of such genetic potential in actinomycetes may lead to the isolation of new biologically active compounds (6–8). We recently described a new method to increase antibiotic production in bacteria by modulating ribosomal components (ribosomal proteins or rRNA), i.e., by introducing mutations conferring drug resistance, as many antibiotics target the ribosome (9–11). This new approach, called "ribosome engineering" (12, 13), has several advantages, including the ability to screen for drug resistance mutations by simple selection on drug-containing plates, even if the mutation frequency is extremely low (e.g., $<10^{-10}$), and the ability to select for mutations without prior genetic information. Hence, this method requires no induced mutagenesis. Interestingly, the introduction of several drug resistance mutations has a cumulative effect on antibiotic production (14–16).

In addition to enhancement of antibiotic production, we have demonstrated that certain *rpoB* mutations which arose in the RNA polymerase (RNAP) β-subunit (and certain *rpsL* mutations which arose in ribosomal protein S12) are effective for activating the "silent" secondary metabolite biosynthetic genes, eventually leading to discovery of novel antibiotics (17–19). Importantly, Derewacz et al. (20) recently reported that drug resistance mutations (e.g., streptomycin resistance or rifampin resistance) affect broad changes in the metabolic phenotype of *Nocardiopsis* sp. in addition to secondary metabolism of this organism. Although earlier work was conducted mainly with *S. coelicolor* A3(2), we have now demonstrated that *rpoB* mutations are widely effective in enhancing the production of antibiotics by various actinomycetes and that *rpoB* mutations activate the secondary metabolite biosynthetic gene clusters, which are "silent" or poorly expressed under ordinary culture conditions. Analysis of the metabolite profile demonstrates that the *rpoB* mutants produced many metabolites, which were not detected in the wild-type strains.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The wild-type strains *S. coelicolor* A3(2), *Streptomyces antibioticus* strain 3720, *Streptomyces parvulus* ATCC 12434, *S. griseus* IFO13189, *Streptomyces lavendulae* MA406A1, *Amycolatopsis orientalis* NBRC12806, and *S. erythraea* NRRL2338 were used. Spontaneous rifampin-resistant mutants were obtained as colonies that grew within 5 to 10 days after spores were spread on GYM agar (21) containing various concentrations of rifampin. Mutations in the *rpoB* gene were determined by DNA sequencing using the primers listed in Table S1 in the supplemental material. Strains were grown in GYM medium (21), SPY medium (21), SYM medium (21), MPY medium (22), R4 medium (23), R3/1 medium (24), or vancomycin production medium (25) at 25°C or 30°C under rotary shaking (220 rpm) as described in the

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TABLE 1 Characterization of rifampin resistance mutations of *Streptomyces griseus* IFO13189, *Streptomyces coelicolor* A3(2), *Saccharopolyspora erythraea* NRRL2338, *Streptomyces antibioticus* 3720, *Streptomyces parvulus* ATCC 12434, *Streptomyces lavendulae* MA406A1, and *Amycolatopsis orientalis* NBRC12806

Strain	Rifampin concn (μg/ml) used for selection	Mutation in *rpoB*[a]	Amino acid substitution	Frequency of mutants with same mutation	Resistance to rifampin (μg/ml)[b]	Concn of antibiotic produced
S. griseus						Streptomycin (μg/ml)[c]
Wild type	NA[d]	NA	NA	NA	0.5	48 ± 14
KO-1167	5	1270C→A	Gln424→Lys	1/30	>200	178 ± 27
KO-1168	5	1275C-1277T→Δ	Phe425,Met426→Leu	1/30	>200	9 ± 1
KO-1169	5	1276A-1278G→Δ	Met426→Δ	1/30	>200	0
KO-1170	5	1280A→T	Asp427→Val	2/30	10	23 ± 5
KO-1171	5	1309C→G	His437→Asp	8/30	50	34 ± 7
KO-1172	5	1309C→T	His437→Tyr	8/30	200	42 ± 9
KO-1173	5	1310A→T	His437→Leu	1/30	10	23 ± 7
KO-1174	5	1310A→G	His437→Arg	1/30	>200	96 ± 4
KO-1175	5	1325C→T	Ser442→Leu	1/30	20	143 ± 16
S. coelicolor						Actinorhodin (OD_{640})[e]
Wild type	NA	NA	NA	NA	20	0.59 ± 0.05 (+)
KO-1123	100	1267T→A	Ser423→Thr	2/32	300	20.7 ± 1.9 (++)
KO-1124	100	1272G→T	Gln424→His	1/32	300	0.88 ± 0.23 (+)
KO-1125	100	1276A→G	Met426→Val	2/32	300	1.46 ± 0.23 (++)
KO-1126	100	1280A→C	Asp427→Ala	1/32	300	19.6 ± 1.5 (++)
KO-1127	100	1280A→G	Asp427→Gly	1/32	>500	0.56 ± 0.15 (+)
KO-1128	100	1280A→T	Asp427→Val	5/32	>500	1.4 ± 0.26 (++)
KO-1129	100	1281C→A	Asp427→Glu	1/32	>500	2.1 ± 0.47 (++)
KO-1130	100	1298C→T	Ser433→Leu	3/32	500	28.7 ± 1.3 (+++)
KO-1131	100	1309C→T	His437→Tyr	3/32	>500	1.3 ± 0.20 (++)
KO-1132	100	1310A→T	His437→Leu	1/32	>500	1.9 ± 0.09 (+++)
KO-1133	100	1319G→A	Arg440→His	3/32	>500	0.31 ± 0.01 (+)
KO-1134	100	1324A→T	Asn442→Tyr	1/32	>500	0.55 ± 0.08 (++)
KO-1135	100	1327G→C	Ala443→Pro	1/32	200	0.37 ± 0.14 (+)
S. erythraea						Erythromycin (μg/ml)[f]
Wild type	NA	NA	NA	NA	0.5	42 ± 7
KO-1194	10	1309C→G	His437→Asp	1/35	>300	88 ± 6
KO-1195	10	1309C→T	His437→Tyr	2/35	>300	90 ± 12
KO-1196	10	1310A→G	His437→Arg	1/35	>300	163 ± 34
KO-1197	10	1318C→T	Arg440→Trp	11/35	200	7 ± 1
KO-1198	10	1318C→G	Arg440→Gly	1/35	200	41 ± 4
KO-1199	10	1319G→T	Arg440→Leu	2/35	80	33 ± 5
KO-1200	10	1319G→A	Arg440→Gln	4/35	30	20 ± 5
KO-1201	10	1325C→A	Ser442→Tyr	1/35	>300	68 ± 18
KO-1202	10	1325C→T	Ser442→Phe	9/35	300	79 ± 11
S. antibioticus						Actinomycin (μg/ml)[g]
Wild type	NA	NA	NA	NA	1	11 ± 2
KO-1212	10	1264C-1275C→Δ	Leu422-Phe425→Δ	1/21	300	2 ± 1
KO-1160	10	1267T-1278G→Δ	Ser423-Met426→Δ	1/21	>300	53 ± 9
KO-1161	10	1270C→G	Gln424→Glu	1/21	50	33 ± 7
KO-1162	10	1309C→G	His437→Asp	2/21	300	9 ± 2
KO-1163	10	1309C→T	His437→Tyr	5/21	>300	79 ± 9
KO-1164	10	1310A→G	His437→Arg	3/21	>300	86 ± 16
KO-1165	10	1319G→A	Arg440→His	1/21	50	23 ± 5
KO-1166	10	1325C→T	Ser442→Leu	1/21	50	19 ± 2
S. parvulus						Actinomycin (μg/ml)[h]
Wild type	NA	NA	NA	NA	20	6 ± 1
KO-1183	100	1261C-1269C→Δ	Gln421-Ser423→Δ	1/23	>300	6 ± 1
KO-1184	100	1279G→A	Asp427→Asn	2/23	>300	6 ± 2
KO-1185	100	1279G→T	Asp427→Tyr	3/23	>300	6 ± 1
KO-1186	100	1280A→G	Asp427→Gly	2/23	>300	7 ± 3
KO-1187	100	1280A→T	Asp427→Val	1/23	>300	10 ± 1
KO-1188	100	1281C-1283T→Δ	Asp427,Gln428→Glu	1/23	>300	6 ± 1
KO-1189	100	1284G-1289A→Δ	Gln428-Asn430→His	1/23	>300	4 ± 1
KO-1190	100	1298C→T	Ser433→Leu	2/23	>300	4 ± 3
KO-1191	100	1309C→A	His437→Asn	1/23	>300	7 ± 1
KO-1192	100	1309C→T	His437→Tyr	4/23	>300	4 ± 1
KO-1193	100	1310A→T	His437→Leu	1/23	>300	6 ± 2
S. lavendulae						Formycin (μg/ml)[i]
Wild type	NA	NA	NA	NA	1	16 ± 1
KO-1176	10	1265T→C	Leu422→Pro	1/23	20	40 ± 7
KO-1177	10	1280A→G	Asp427→Gly	6/23	20	0
KO-1178	10	1298C→T	Ser433→Leu	1/23	30	0
KO-1179	10	1309C→T	His437→Tyr	4/23	200	0
KO-1180	10	1309C→G	His437→Asp	1/23	200	8 ± 1
KO-1181	10	1310A→G	His437→Arg	1/23	300	0
KO-1182	10	1319G→A	Arg440→His	1/23	50	55 ± 14

(Continued on following page)

TABLE 1 (Continued)

Strain	Rifampin concn (μg/ml) used for selection	Mutation in *rpoB*[a]	Amino acid substitution	Frequency of mutants with same mutation	Resistance to rifampin (μg/ml)[b]	Concn of antibiotic produced
A. orientalis						Vancomycin (μg/ml)[j]
Wild type	NA	NA	NA	NA	20	90 ± 7
KO-1153	30	1271A→T	Gln424→Leu	1/84	>300	19 ± 1
KO-1154	30	1309C→T	His437→Tyr	22/84	>300	172 ± 7
KO-1155	30	1310A→G	His437→Arg	29/84	>300	80 ± 5
KO-1156	30	1319G→T	Arg440→Leu	1/84	>300	0
KO-1157	30	1319G→A	Arg440→His	7/84	>300	0
KO-1158	30	1325C→A	Ser442→Tyr	1/84	>300	270 ± 17
KO-1159	30	1325C→T	Ser442→Phe	23/84	>300	101 ± 5

[a] Numbered from the start codon of the open reading frame of *S. coelicolor*.
[b] Determined after 5 days of incubation on GYM medium.
[c] Strains were grown in SPY medium at 25°C for 3 days. All measurements were performed in triplicate.
[d] NA, not applicable (parent strain).
[e] Results for actinorhodin were determined after 5 days of incubation in GYM liquid medium or 7 days of incubation on GYM agar plate. Shown are mean values of optical density at 640 nm (OD_{640}) of triplicate flasks (in liquid culture). +, poor production; ++, moderate; +++, abundant (in solid culture).
[f] Strains were grown in R3/1 medium at 30°C for 6 days. All measurements were performed in triplicate.
[g] Strains were grown in SYM medium at 30°C for 5 days. All measurements were performed in triplicate.
[h] Strains were grown in 2× SYM medium at 30°C for 6 days. All measurements were performed in triplicate.
[i] Strains were grown in MPY medium at 25°C for 2 days. All measurements were performed in triplicate.
[j] Strains were grown in vancomycin production medium at 30°C for 6 days. All measurements were performed in triplicate.

text. All measurements for antibiotic productivity were performed in triplicate or more flasks, and the antibiotic titers were expressed as means ± standard deviations (SD).

Assays for antibiotics. Actinorhodin produced in liquid culture was determined by the method of Kieser et al. (26). The level of actinorhodin production on the plates was assessed directly by determination of the intensity of the blue color. Actinomycin was determined photometrically as described previously (11). Streptomycin, formycin, and erythromycin were determined by bioassay (agar diffusion method) using test organisms as described previously (11, 21, 22). Vancomycin was determined by bioassay using *Staphylococcus aureus* 209P as a test organism.

Determination of MICs. MICs were determined by spotting spore solutions (~10^6) onto rifampin-containing GYM plates, followed by incubation at 30°C for the indicated times. The minimum drug concentration able to fully inhibit growth was defined as the MIC.

Transcriptional analysis by real-time qPCR. Total RNAs were extracted and purified from cells grown for the indicated times, using Isogen reagent (Nippon Gene) according to the manufacturer's protocol. Real-time quantitative PCR (qPCR) was performed as described previously (27). Each transcription assay was normalized relative to the corresponding transcriptional level of *hrdB* (for *S. griseus* and *S. coelicolor*) or *sigB* (for *S. erythraea*), a gene encoding the principal sigma factor. The primers used for real-time qPCR are listed in Table S1 in the supplemental material.

Conjugation procedure. Conjugation-based gene transfer between *S. coelicolor* strains was performed as described by Kieser et al. (26), using the *actII*-open reading frame 4 (ORF4) disruptant (with hygromycin resistance). For transfer of the disrupted *actII*-ORF4 gene between the disruptant and the *rpoB* mutant KO-1130, conjugants were selected for rifampin resistance and hygromycin resistance. Mutations were confirmed by DNA sequencing.

Analysis of metabolites by UPLC/MS. A double volume of acetonitrile was added to the culture broth and mixed well. The mixture was then centrifuged at 15,000 × *g* for 1 min, and the supernatant was analyzed directly by ultraperformance liquid chromatography-mass spectrometry (UPLC/MS). The analytical conditions were as follows: device, Waters Acquity UPLC H-class; column, Waters Acquity UPLC ethylene bridged hybrid (BEH) C_{18} (2.1 by 150 mm; particle size, 1.7 μm); column temperature, 40°C; gradient elution, solvent A (acetonitrile), solvent B (MeOH), solvent C (0.1% CH_3COONH_4 in deionized water), solvent D (deionized water); gradient profile, 0 to 1 min, 20% A, 20% to 60% B, 20% C, 40% to 0% D; 1 to 6 min, 20% A, 60% B, 20% C, 0% D; 6 to 6.5 min, 20% A, 60% to 20% B, 20% C, 0% to 40% D; 6.5 to 9.0 min, 20% A, 20% B, 20% D, 40% D; flow rate, 0.25 ml/min; detection, *m/z* between 200 and 800 (for *S. coelicolor*) or between 200 and 500 (for *S. griseus* and *S. erythraea*) using a Waters SQ detector mass spectrometer; ionization mode, electrospray ionization (ESI) positive, capillary voltage, 3.3 kV; source temperature, 120°C; desolvation temperature, 350°C; desolvation gas flow, 600 liters/h; cone gas flow, 50 liters/h; cone voltage, 10 V for *S. griseus*, 60 V for *S. coelicolor*, and 40 V for *S. erythraea* metabolite analyses.

RESULTS

Isolation and characterization of rifampin-resistant mutants. To investigate the effects of *rpoB* mutations in various actinomycetes, we isolated a number of spontaneous rifampin-resistant (*rif*) mutants. When spores of strains were spread and incubated on GYM agar containing various concentrations of rifampin, *rif* mutants developed after 5 to 10 days at a frequency of 10^{-7} to 10^{-8}. The sequence of the *rpoB* gene, which encodes the RNAP β-subunit, was determined by DNA sequencing analysis, and the majority of the mutants tested had a mutation in the so-called "*rif* cluster" of the *rpoB* gene (we analyzed 21 to 84 *rif* mutants for each actinomycete) (Table 1). Strikingly, the introduction of certain *rpoB* mutations effectively increased antibiotic production by *S. griseus* (streptomycin producer), *S. coelicolor* (actinorhodin producer), *S. antibioticus* (actinomycin producer), *S. lavendulae* (formycin producer), *S. erythraea* (erythromycin producer), and *A. orientalis* (vancomycin producer). These antibiotics are characterized structurally as polyketides, polyethers, glycopeptides, macrolides, polypeptides, nucleotides, and aminoglycosides. Only *S. parvulus* (actinomycin producer) showed essentially no increase in antibiotic production in any media examined, including 2× SYM medium, although actinomycin production by *S. antibioticus* was markedly enhanced by introducing certain *rpoB* mutations (Table 1). The *rpoB* mutations that yielded antibiotic overproduction are summarized in Table 2. All *rpoB* mutations detected in this study were located within positions 1264C to 1327G, representing amino acid residues Leu422 to Ala443. Importantly, *rpoB* H437Y (altering His at position 437 to Tyr) and H437R (altering His at position 437 to Arg) mutations were most often effective in a wide variety of actinomycetes. It is also notable that alteration of Ser at position 433 or 442 was also often effective,

TABLE 2 Summary of *rpoB* mutations effective for antibiotic overproduction

Microorganism	Antibiotic	Effective *rpoB* mutation(s)[a]	Source or reference
S. lividans	Actinorhodin	S433P, S433L, **H437R**, **H437Y**, R440C, R599C	19, 40
S. mauvecolor	Piperidamycin	H437D, H437L, **H437R**, **H437Y**	18
S. albus	Salinomycin	S442F	14
S. incarnatus	Sinefungin	D427G	49
S. coelicolor	Actinorhodin	S423T, D427A, S433L, S433P, H437L, **H437R, H437Y**	42; this study
S. griseus	Streptomycin	Q424K, **H437R**, S442L	This study
S. antibioticus	Actinomycin	Q424E, **H437R**, **H437Y**	This study
S. parvulus	Actinomycin	D427V	This study
S. lavendulae	Formycin	L422P, R440H	This study
A. orientalis	Vancomycin	**H437Y**, S442Y	This study
S. erythraea	Erythromycin	H437D, **H437R**, **H437Y**, S442F, S442Y	This study

[a] Mutations are numbered from the start codon of the open reading frame of *S. coelicolor*. Boldface characters show mutations that were often detected as antibiotic overproduction mutations.

although position 442 is variable among species (e.g., Asn in *S. coelicolor*).

Effects of *rpoB* mutations on streptomycin biosynthetic genes in *S. griseus*. We reported previously that *rsmG* mutations conferring a low level of resistance to streptomycin cause antibiotic overproduction in *S. coelicolor* and *S. griseus* and that enhanced expression of the *metK* gene encoding *S*-adenosylmethionine synthetase is responsible for the enhanced production of actinorhodin and streptomycin (10, 23). In fact, addition of *S*-adenosylmethionine causes overproduction of streptomycin in *S. griseus*, accompanied by enhanced expression of *adpA* (encoding a transcriptional regulator) and *strR* (encoding the streptomycin biosynthesis operon regulator). As the Q424K mutation in *rpoB* was most effective in enhancing streptomycin production (Table 1), we analyzed transcription of *metK*, *adpA*, and *strR*, together with several genes (*strB1*, *strF*, and *strD*) involved in streptomycin biosynthesis. As expected, expression of *strR* was markedly enhanced in the *rpoB* Q424K mutant KO-1167 compared to the wild-type strain at both early (9-h) and late (24-h) growth phases, accompanied by enhanced expression of biosynthetic genes (*strB1*, *strD*, and *strF*), underlying the enhanced production of streptomycin in KO-1167 (Fig. 1). In contrast, expression of *metK* was marginally increased only in the early growth phase, and expression of *adpA* was rather impaired. Thus, unlike the case for *rsmG* mutant, enhanced expression of *strR* appears to be solely responsible for the observed streptomycin overproduction in the *rpoB* Q424K mutant, although AdpA acts as a central transcriptional regulator in the A-factor regulatory cascade in the *S. griseus* wild-type strain (28).

Similarly, the biosyntheses of actinorhodin (in *S. coelicolor*) and erythromycin (in *S. erythraea*) are controlled by transcription regulatory proteins ActII-ORF4 and BldD, respectively (29, 30). Notably, *rpoB* mutants (KO-1130 and KO-1131 of *S. coelicolor* and KO-1195 and KO-1196 of *S. erythraea*) with enhanced production of actinorhodin and erythromycin displayed enhanced expression of *actII*-ORF4 and *bldD*, respectively, accounting for the increased antibiotic production in these *rpoB* mutants (Fig. 2B). In accordance with the upregulation of *bldD*, expression of *eryCII* (a gene for erythromycin biosynthesis) increased 2- to 5-fold in the *rpoB* H437Y mutant of *S. erythraea* (data not shown). On the other hand, expression of another transcription regulatory gene, *redD* (controlling the biosynthesis operon for undecylprodigiosin in *S. coelicolor*), was not upregulated but rather downregulated, showing differential effects of each *rpoB* mutation on the expression of each transcription regulatory gene (Fig. 2B).

***rpoB* mutations widely activate transcription of cryptic secondary metabolite biosynthetic gene clusters.** A recent study in our laboratory indicated that certain mutations in *rpoB* or *rpsL* genes can activate silent or weakly expressed genes of actinomycetes or *Bacillus subtilis*, leading to the discovery of novel antibacterial agents (17, 18). Later, we found that *rsmG* mutations, which confer a low level of resistance to streptomycin, can activate not only streptomycin production but also the expression of other secondary metabolite biosynthetic genes in *S. griseus* (31). Therefore, we turned our attention to the effects of *rpoB* mutations on

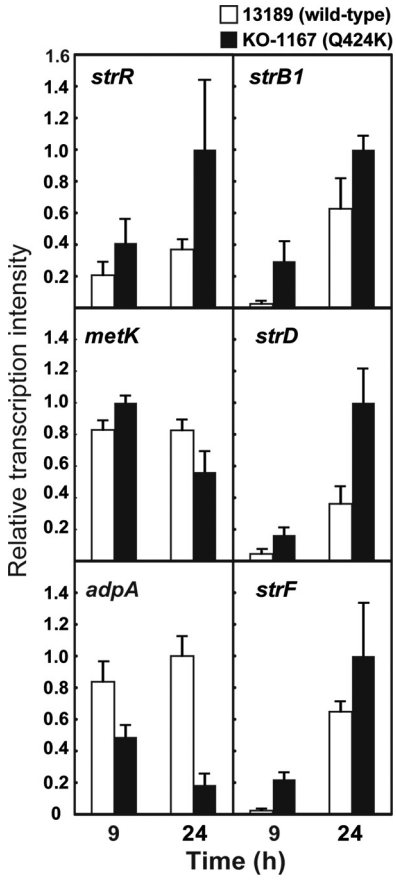

FIG 1 Transcriptional analysis of *strR*, *metK*, *adpA*, *strB1*, *strD*, and *strF* by real-time qPCR in *S. griseus*. The RNAs were extracted from cells of wild-type (13189) and KO-1167 (*rpoB* Q424K) mutant strains grown at 25°C to the early (9 h) or late growth phase (24 h) in SPY medium. Total RNA preparation and real-time qPCR were performed as described in Materials and Methods. The maximum expression levels were taken as unity (= 1). The error bars indicate the standard deviations of the means of three or more samples.

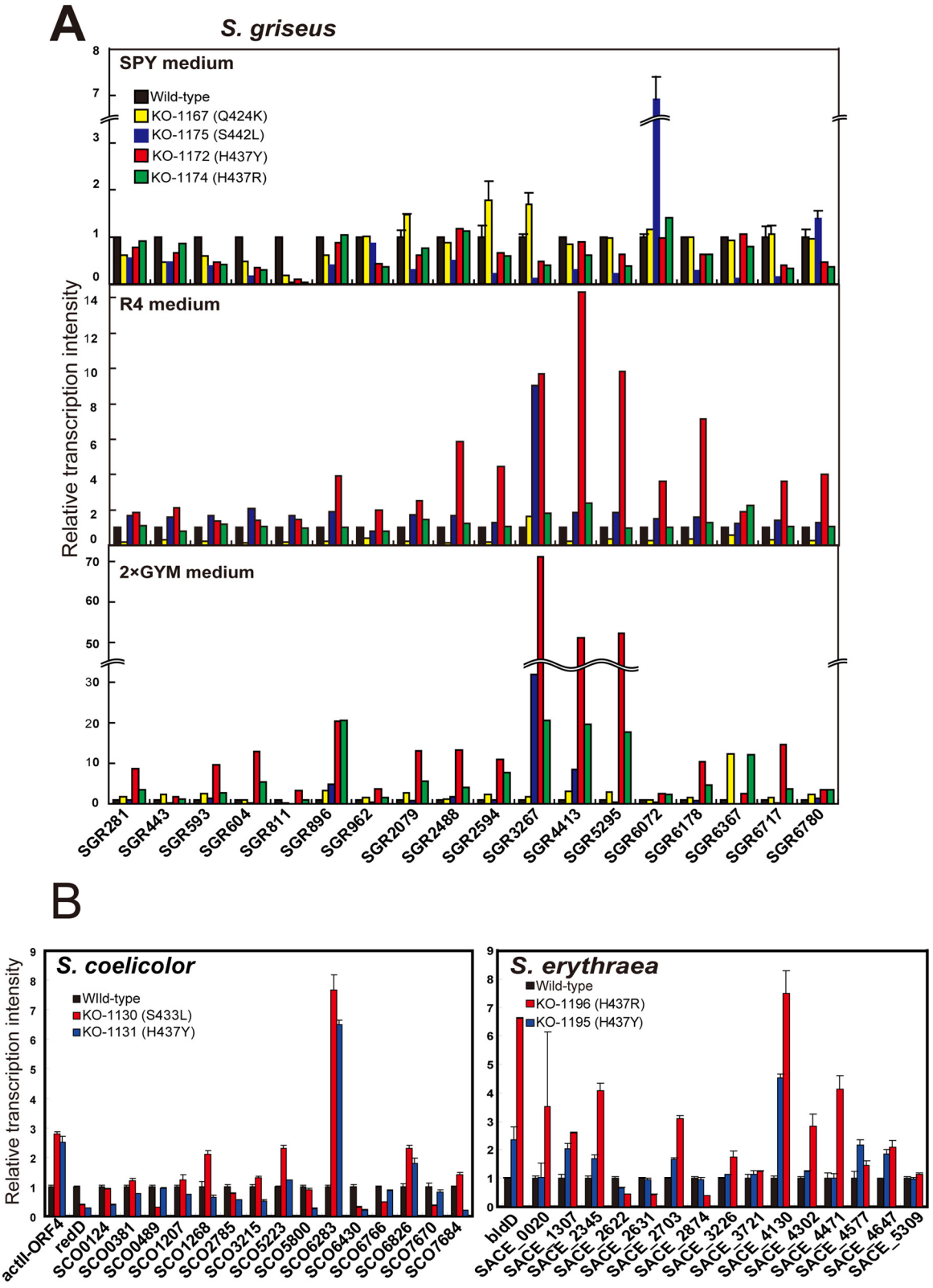

FIG 2 Transcriptional analysis of the genes involved in the secondary metabolite biosynthetic gene clusters in the *rpoB* mutant strains of *S. griseus*, *S. coelicolor*, and *S. erythraea*. Total RNA preparation and real-time qPCR were performed as described in Materials and Methods. The maximum expression levels detected in each culture were compared, taking the maximum expression levels of the wild-type strain as unity (= 1). (A) The RNAs were extracted from *S. griseus* cells grown to the late growth phase (24 h in SPY medium at 25°C; 24, 36, 48, and 60 h in R4 medium at 30°C; 12, 24, and 36 h in 2× GYM medium at 30°C). (B) The RNAs were extracted from cells grown at 30°C to the late growth phase (24, 36, and 48 h in GYM medium for *S. coelicolor*; 12, 24, 36, and 48 h in 2× GYM medium for *S. erythraea*).

the expression of cryptic secondary metabolite biosynthetic gene clusters in various actinomycetes to assess the broad applicability of the *rpoB* mutation method. First, a total of 18 genes belonging to 18 secondary metabolite biosynthetic gene clusters of *S. griseus* (Table 3) were subjected to transcriptional analysis by real-time quantitative PCR (qPCR), comparing the wild-type and *rpoB* mutant (Q424K, S442L, H437Y, and H437R) strains. The RNAs were extracted from cells grown to late growth phase, and the maximum expression levels detected in each culture were compared (Fig. 2A). The profiles of changes in expression of each gene are shown in Fig. S1 and S2 in the supplemental material. Strikingly, of the *rpoB* mutants examined, strain KO-1172 with the H437Y mutation grown in 2× GYM medium showed remarkable activation of cryptic genes at the transcriptional level. This was especially pronounced for SGR3267, SGR4413, and SGR5295, which were activated by 50- to 70-fold. Likewise, the H437Y mutation was effective when cells were grown in R4 medium (activated up to 14-fold), while the efficacy of the H437Y mutation was no longer detected when cells were grown in SPY medium (a medium developed for streptomycin production). Although the H437Y mutation was effective in both 2× GYM medium and R4 medium, the H437R mutation exerted its effect only in R4 medium. It is notable that although the Q424K and S442L mutations were quite effective at enhancing streptomycin production (Table 1), these mutations were not effective in activating the cryptic genes, except for SGR3267, SGR6072, and SGR6367, which were activated by 7- to 30-fold (Fig. 2A). These results indicated that the abilities of *rpoB* mutations to activate the cryptic genes are medium dependent, with each *rpoB* mutation exerting differential effects on the activation of each cryptic gene cluster.

Next, we analyzed cryptic gene activation in *S. coelicolor* and *S. erythraea*. A total of 15 genes belonging to 15 secondary metabolite biosynthetic gene clusters (Table 3) were subjected to transcription analysis using cells grown to the late growth phase. As expected, the H437Y and H437R mutations, which were effective in enhancing erythromycin production (Table 1), were widely effective in enhancing the activity of the cryptic genes of *S. erythraea*; 6 of the 15 genes examined showed a 3-fold or more increase in transcription (Fig. 2B). Similarly, the S433L and H437Y mutations, which were effective in enhancing actinorhodin production (Table 1), were effective, although not remarkable, in enhancing the activity of the cryptic genes of *S. coelicolor*. The profiles of changes in expression of each gene are shown in Fig. S3 (for *S. coelicolor*) and S4 (for *S. erythraea*) in the supplemental material.

The *S. griseus* cryptic genes SGR3267 and SGR5295 cannot be activated under general stress conditions. It is widely accepted that bacterial secondary metabolism usually starts when cells encounter adverse environmental conditions, as represented by nutrient limitation or the presence of stress stimuli. As the *S. griseus* cryptic genes SGR3267 and SGR5295 were markedly activated by introducing certain *rpoB* mutations, we were interested in whether these cryptic genes can be activated under certain stressful conditions. Therefore, we conducted transcriptional analysis of SGR3267 and SGR5295 using cells that had been subjected to 30 different stress conditions, followed by a further 3 h of incubation (Table 4). These stress stimuli included pH changes, temperature shifts, and addition of chemicals, such as heavy metals, antibiotics, and flavonoids. As heavy metals and antibiotics are potent growth inhibitors, these chemicals were added at sublethal concentrations (i.e., one-third of the MIC). Despite the wide variety of stress conditions, these genes were not activated under the stress conditions examined. Treatment with rifampin was also ineffective. These results can be taken as evidence that the genes SGR3267 and SGR5295 are "silent" under laboratory fermentation conditions and, in turn, emphasize the efficacy of *rpoB* mutations that resulted in 50- to 70-fold activation.

We reported previously that rare earth elements, such as scandium (Sc) and lanthanum (La), not only enhance antibiotic production but also activate expression of the cryptic secondary metabolite biosynthetic gene clusters in *S. coelicolor* (27). Strikingly, scandium and lanthanum exerted their apparent effects on transcription of SGR3267 and SGR5295 of *S. griseus* when added to the medium at low concentrations. Transcription levels of these genes were upregulated by 4-fold with treatment of the cells with a sublethal concentration (one-third of the MIC) of lanthanum (Table 4), thus highlighting rare earth elements as distinguished stress stimuli.

The *rpoB* mutants produce many metabolites not detected in the wild-type strain. (i) Metabolite analysis with 2D-UPLC/MS. As the *rpoB* mutations markedly upregulated the expression of cryptic genes at the transcriptional level, we compared metabolic profiles between the wild-type and *rpoB* mutant strains. Strains of *S. griseus*, *S. coelicolor*, and *S. erythraea* were grown to the late growth phase, and then the metabolites produced were analyzed by two-dimensional (2D)-UPLC/MS. Strikingly, the *rpoB* mutants produced many metabolites that were not detected in the wild-type strains. This was especially pronounced in *S. griseus* KO-1172 (H437Y) and *S. erythraea* KO-1195 (H437Y) and KO-1196 (H437R), as indicated by red circles in Fig. 3A. For quantitative description, we calculated each peak area from selected ion chromatograms and compared the peak areas among the wild-type and *rpoB* mutant strains (Fig. 3B). In *S. griseus*, the levels of production of metabolites designated zones no. 3 (*m/z* 400) and no. 5 (*m/z* 358) were markedly activated, and zones no. 6 (*m/z* 299) and no. 7 (*m/z* 299) showed extensive activation. In *S. coelicolor*, the production levels of metabolites designated zone no. 1 (*m/z* 631, 633, 647, and 649) were remarkably activated, and that of zone no. 3 (*m/z* 268) was extensively activated. The metabolites with *m/z* of 631, 633, and 649 were assigned as γ-actinorhodin, γ′-actinorhodin, and ε-actinorhodin, respectively (26, 32, 33), while the metabolite with *m/z* 647 was considered a novel actinorhodin-related compound, because disruption of *actII*-ORF4 in KO-1130 resulted in almost complete abolition of these four metabolites simultaneously (see Fig. S5A in the supplemental material). The structures of these known actinorhodin-related compounds, together with the possible structure of the unknown actinorhodin, are shown in Fig. S5B.

Quantitative comparison of metabolites in *S. erythraea* strains was characterized by marked activation of the production of many metabolites (Fig. 3B), as expected from the results of 2D-UPLC/MS (Fig. 3A).

(ii) Differential effects of *rpoB* mutations on metabolite profile. Although the results shown in Fig. 3B suggest differential effects of *rpoB* mutations on metabolite profile, this was confirmed by analyzing the metabolite profiles of nine *S. griseus rpoB* mutants. As shown in Fig. 4, it is apparent that the best *rpoB* mutation to enhance the yield of each metabolite varies from metabolite to metabolite, although the H437Y mutation was most often the best. For example, the H437D mutation was effective for productivity enhancement of the metabolites designated zone no.

TABLE 3 Secondary metabolite biosynthetic genes of *S. griseus* IFO13189, *S. coelicolor* A3(2), and *S. erythraea* NRRL2338 analyzed in this study

Organism and gene[a]	Product(s)	Secondary metabolite biosynthetic gene cluster product(s)[b]
S. griseus		
metK	*S*-Adenosylmethionine synthetase	—[c]
adpA	Transcriptional regulator	—
strR	Streptomycin biosynthesis operon regulator	Streptomycin
strB1	Scyllo-inosamine-4-phosphate amidinotransferase	Streptomycin
strD	Putative glucose-1-phosphate thymidylyltransferase	Streptomycin
strF	StrF protein	Streptomycin
SGR281	Hypothetical protein	PKS-NRPS hybrid (SGR278–SGR283)
SGR443	Putative ABC transporter ATPase and permease component	NRPS for siderophore (SGR443–SGR455)
SGR593	Hypothetical protein	NRPS (SGR574–SGR593)
SGR604	Putative enediyne biosynthesis protein	Enediyne PKS (SGR604–SGR611)
SGR811	Putative oxidoreductase	PKS-NRPS hybrid (SGR810–SGR815)
SGR896	Putative *O*-methyltransferase	NRPS (SGR895–SGR901)
SGR962	Putative squalene-hopene cyclase	Hopanoid (SGR962–SGR966)
SGR2079	Putative terpene cyclase	Terpene (SGR2079)
SGR2488	Putative dehydrogenase	Type I PKS, NRPS (SGR2482–SGR2489)
SGR2594	Putative integral membrane ion antiporter	NRPS (SGR2586–SGR2598)
SGR3267	Putative cytochrome P450	Type II PKS, NRPS (SGR3239–SGR3288)
SGR4413	Putative lantibiotic biosynthesis protein	Lantibiotic (SGR4408–SGR4421)
SGR5295	5-Aminolevulinate synthase	Unknown (SGR5285–SGR5295)
SGR6072	Putative ketosteroid isomerase	Type I PKS (SGR6071–SGR6083)
SGR6178	Putative thioesterase	Type I PKS (SGR6177–SGR6183)
SGR6367	Putative oxidoreductase	Type I PKS (SGR6360–SGR6387)
SGR6717	Putative ABC transporter ATPase and permease component	NRPS for siderophore (SGR6709–SGR6717)
SGR6780	Putative malonyl-coenzyme A:ACP transacylase	Type I PKS, PKS-NRPS hybrid (SGR6776–SGR6786)
S. coelicolor		
SCO0124	Hypothetical protein	Eicosapentaenoic acid (type I iterative PKS; SCO0124–SCO0129)
SCO0381	Putative glycosyl transferase	Unknown (deoxysugar; SCO0381–SCO0401)
SCO0489	Conserved hypothetical protein	Coelichelin (NRPS; SCO0489–SCO0499)
SCO1207	Putative cytochrome P450	Tetrahydroxynaphthalene (chalcone synthase; SCO1206–SCO1208)
SCO1268	Putative acyltransferase	Unknown (type II fatty acid synthase; SCO1265–SCO1273)
SCO2785	Conserved hypothetical protein	Desferrioxamines (siderophore synthetase; SCO2782–SCO2785)
SCO3215	Hypothetical protein	CDA[d] (NRPS; SCO3210–SCO3249)
SCO5085 (*actII*-ORF4)	Actinorhodin cluster activator protein	Actinorhodin (type II PKS; SCO5071–SCO5092)
SCO5223	Putative cytochrome P450	Unknown (sesquiterpene synthase; SCO5222–SCO5223)
SCO5800	Conserved hypothetical protein	Unknown (siderophore synthetase; SCO5799–SCO5801)
SCO5877 (*redD*)	Transcriptional regulator RedD	Prodiginines (NRPS, type I modular PKS; SCO5877–SCO5898)
SCO6283	Conserved hypothetical protein	Unknown (type I modular PKS; SCO6273–SCO6288)
SCO6430	Hypothetical protein	Unknown (NRPS; SCO6429–SCO6438)
SCO6766	Conserved hypothetical protein	Hopanoids (squalene-hopene cyclase; SCO6759–SCO6771)
SCO6826	Conserved hypothetical protein	Unknown (type I modular PKS; SCO6826–SCO6827)
SCO7670	Conserved hypothetical protein	Unknown (chalcone synthase; SCO7669–SCO7671)
SCO7684	Conserved hypothetical protein	Coelibactin (NRPS; SCO7681–SCO7691)
S. erythraea		
bldD	Putative transcriptional regulator	—
SACE_0020	Hypothetical protein	Pfa (polyketides; SACE_0018–SACE_0028)
SACE_1307	Hypothetical protein	Nrps1 (nonribosomal peptides; SACE_1304– SACE_1310)
SACE_2345	Putative hydrolase	Pks1 (polyketides; SACE_2342–SACE_2347)
SACE_2622	Cytochrome P450	Nrps2-Pks (nonribosomal peptides; SACE_1304–SACE_1310)
SACE_2631	Cytochrome P450-like enzyme	Pks2 (polyketides; SACE_2628–SACE_2631)
SACE_2703	Hypothetical protein	Nrps3 (nonribosomal peptides; SACE_2692–SACE_2703)
SACE_2874	Phospho-2-dehydro-3-deoxyheptonate aldolase	Pks3 (polyketides; SACE_2864–SACE_2879)
SACE_3226	Hypothetical protein	Nrps6 (nonribosomal peptides; SACE_3223–SACE_3229)
SACE_3721	Methyltransferase	Tpc2 (terpens; SACE_3721–SACE_3723)
SACE_4130	Hypothetical protein	Pke (polyketides; SACE_4128–SACE_4145)
SACE_4302	Hypothetical protein	Pks4 (polyketides; SACE_4302–SACE_4307)
SACE_4471	Hypothetical protein	Pks5 (polyketides; SACE_4471–SACE_4478)
SACE_4577	Hypothetical protein	Pks6 (polyketides; SACE_4567–SACE_4578)
SACE_4647	UbiA prenyltransferase	Tpc4 (terpens; SACE_4645–SACE_4654)
SACE_5309	Putative cytochrome P450	Pks7 (polyketides; SACE_5306–SACE_5309)

[a] Gene names are from Ohnishi et al. (4), Bentley et al. (2), and Oliynyk et al. (5).

[b] Obtained from Ohnishi et al. (4), Challis and Hopwood (50), and Oliynyk et al. (5). PKS, polyketide synthase; NRPS, nonribosomal peptide synthetase; CDA, calcium-dependent antibiotic.

[c] —, not a secondary metabolite biosynthetic gene cluster.

TABLE 4 Transcriptional analysis of the *S. griseus* genes SGR3267 and SGR5295 under various stressful conditions[a]

Treatment	Relative expression level of[b]:	
	SGR3267	SGR5295
Control (no treatment)	1	1
H_2O_2 (0.1%)	0.6	0.6
Oxygen limitation (no shaking)	1.1	0.6
Acidified (pH 5)	1.1	1.4
Alkalinized (pH 10)	0.6	0.7
High temp (37°C)	1.2	1.1
Low temp (16°C)	0.9	1.8
Ethanol (2%)	0.7	0.6
Sorbitol (10%)	0.3	0.5
NaCl (10%)	0.2	0.3
2-Deoxy-D-glucose (2%)	0.6	0.7
Serine hydroxamate (2 mM)	0.6	0.7
EDTA (10 mM)	0.5	0.4
$CuSO_4$ (1 mM)	1.5	1.2
$ZnSO_4$ (3 mM)	1.8	1.5
$CoCl_2$ (0.3 mM)	0.9	1.9
$MnCl_2$ (10 mM)	1.0	1.7
$Ga_2(SO_4)$ (1 mM)	0.7	1.4
DMSO[c] (0.2%)	0.7	0.5
Decoynine (0.3 mM)	0.7	1.1
Rifampin (0.5 μg/ml)	0.9	0.8
Rifampin (0.17 μg/ml)	0.8	0.7
Gentamicin (0.3 μg/ml)	1.1	0.6
Penicillin (10 μg/ml)	0.8	0.5
Vancomycin (0.3 μg/ml)	0.7	0.5
Caffeine (50 mM)	1.6	1.1
Quercetin (3 mM)	1.6	0.6
Kaempferol (2.3 mM)	0.4	0.5
Apigenin (1.23 mM)	0.4	0.6
Luteolin (2.3 mM)	0.8	0.8
Scandium chloride (0.5 mM)	**3.0**	**2.8**
Lanthanum chloride (1 mM)	**4.1**	**4.2**

[a] *S. griseus* IFO13189 (wild-type strain) was grown in 2× GYM medium at 30°C for 12 h. Then the cells were subjected to each stress treatment (addition of chemicals, pH changes, or temperature shift), followed by a further 3 h of incubation. RNAs were extracted as described in Materials and Methods. The results with the rare earth elements are shown in boldface.

[b] The expression levels are shown taking the expression level of the control (no treatment) as unity (= 1).

[c] DMSO, dimethyl sulfoxide.

3 but ineffective for zones no. 6 and 7. Similarly, the Q424K mutation was effective for zone no. 1 but ineffective for other metabolites.

DISCUSSION

Genome sequencing of *Streptomyces*, fungi, and myxobacteria showed that, although each strain contains genes encoding the enzymes necessary to synthesize a plethora of potential secondary metabolites, only a fraction are expressed during fermentation. There has been increasing interest in the activation of these cryptic pathways. In this study, we demonstrated that *rpoB* mutations are widely effective not only in enhancing antibiotic production but also in activating the silent genes at both the transcriptional and metabolite levels in various actinomycetes. Therefore, this approach may solve the early stage discovery problems of (i) inducing some level of expression of cryptic biosynthetic gene clusters ("waking" the sleeping genes) and (ii) rapidly increasing product yields to obtain enough material to characterize chemically and biologically (early stage yield enhancement) (34). It is notable that the activation of silent gene clusters by *rpoB* mutations at the transcriptional level was medium dependent, with each *rpoB* mutation exerting differential effects on the activation of each silent gene cluster. Conceivably, the expression of each silent gene cluster is controlled by multiple factors, which are affected, qualitatively and quantitatively, under various culture conditions. These findings suggest that strains containing different *rpoB* mutations (e.g., H437Y and H437R) should be grown in different media to access the full spectrum of silent gene activation. It is also notable that the *rpoB* mutations were effective in enhancing actinomycin production in *S. antibioticus* but not *S. parvulus*, indicating the strain dependency of the *rpoB* mutation effect. Similarly, erythromycin resistance (*ery*) mutations were effective in enhancing actinomycin production in *S. parvulus* but not *S. antibioticus*, although the reason for this difference remains unknown (35).

The genetic regulation of streptomycin biosynthesis has been studied in detail in relation to A-factor (28). Briefly, ArpA (A-factor receptor protein) negatively regulates the expression of *adpA* in the absence of A-factor. The resulting transcriptional activator, AdpA, synthesized in the presence of A-factor, positively regulates the expression of *strR*, leading to synthesis of StrR. This StrR protein switches on expression of the streptomycin biosynthesis operon as a pathway-specific regulator protein (36). The present study clearly demonstrated a detrimental effect of the *rpoB* Q424K mutation on *adpA* expression (Fig. 1). Nevertheless, the expression of *strR* was markedly upregulated, resulting in enhanced expression of biosynthetic genes (*strB1*, *strD*, and *strF*) and eventual streptomycin overproduction. Mutant RNAP with the Q424K mutation was apparently more favorable than the native RNAP in transcribing *strR*. These results, in turn, showed that modulation of downregulated genes (as represented by pathway-specific regulatory genes *strR* in streptomycin production and *actII*-ORF4 in actinorhodin production) is more essential in subjecting the *rpoB* mutations to antibiotic overproduction. *S. erythraea* has been widely studied as a model system for antibiotic production (37, 38). Interestingly, BldD, a key developmental regulator in actinomycetes (30), regulates the synthesis of erythromycin (39). In accordance with this previous finding, the *rpoB* H437R mutation caused a marked (6.5-fold) enhancement of *bldD* expression, eventually leading to upregulation of a biosynthetic gene (*eryCII*) and erythromycin overproduction (Fig. 2B, Table 1). As discussed by Carata et al. (24), the increased erythromycin production may also be due, in part, to the effects of *rpoB* H437R on the expression of genes encoding key enzymes involved in carbon and nitrogen metabolism, which may result in activation of erythromycin feeder pathways. The recent study of Derewacz et al. (20) working with *Nocardiopsis* sp. supports this notion. In turn, although we did not analyze in the present study the *rpoB* mutants with reduced ability to produce antibiotics, it is possible that mutant RNAPs with such *rpoB* mutations may have a reduced affinity to the promoter region of transcription regulatory genes for secondary metabolite biosynthetic genes or may perturb the expression of key genes for primary metabolism, which may result in deactivation of antibiotic feeder pathways.

The introduction of the *rpoB* mutation S487L into a *B. subtilis* strain resulted in cells that overproduced an amino sugar antibiotic, 3,3′-neotrehalosadiamine (NTD), the production of which is dormant in the wild-type strain (17). Unlike the wild-type RNAP,

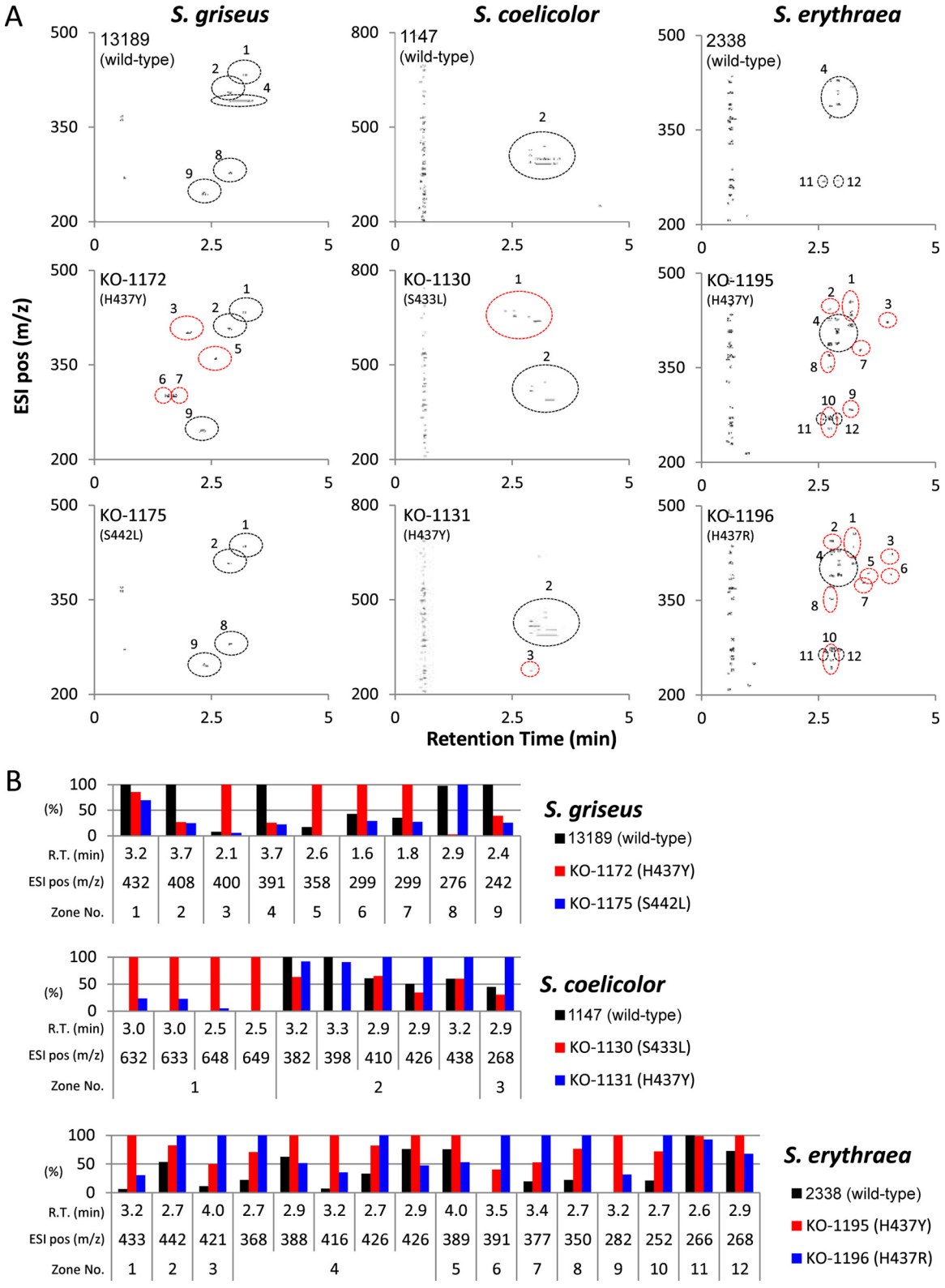

FIG 3 Analysis of the metabolite profile derived from the *rpoB* mutants. The strains were grown for 4 days in 2× GYM medium (for *S. griseus*) or GYM medium (for *S. coelicolor*) as described in the legend to Fig. 2. *S. erythraea* strains were grown for 2 days in 2× GYM medium. The metabolite analysis was performed as described in Materials and Methods. (A) Metabolite profiles of wild-type and *rpoB* mutant strains as analyzed by 2D-UPLC/MS. The results are shown as 2D plots (positive [pos] ions [*m/z*] versus retention time [min]). The spots detected in the wild type are marked by black circles, while those not detected in the wild type are marked by red circles. (B) Comparison of peak area of metabolites produced by wild-type and *rpoB* mutant strains. The differences in peak areas were calculated from selected ion chromatograms. The largest peak area was designated 100%. R.T., retention time.

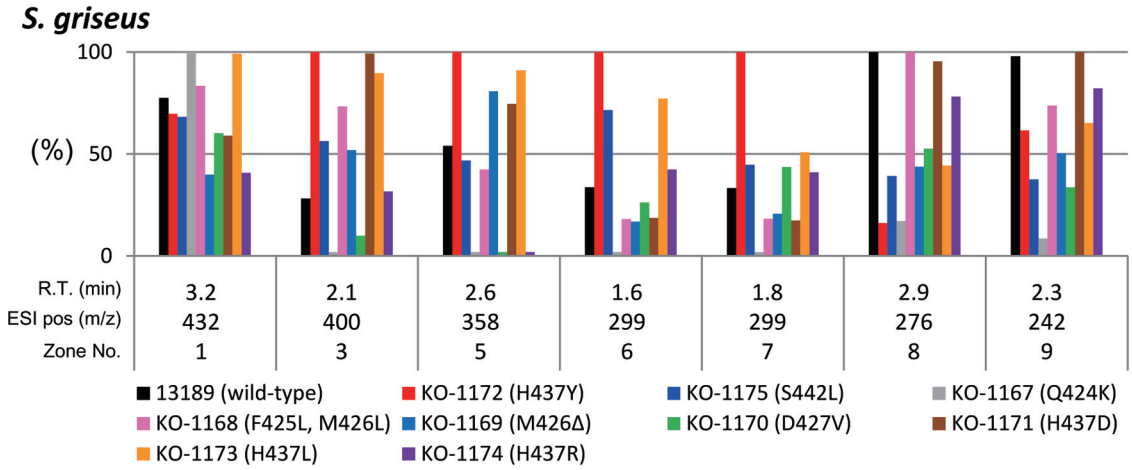

FIG 4 Differential effects of *rpoB* mutations on the metabolite profile in *S. griseus.* Nine *rpoB* mutants were grown as described in the legend to Fig. 3, and the metabolites were analyzed by 2D-UPLC/MS. The peak areas calculated from selected ion chromatograms are shown, taking the largest peak area as unity (100%).

it is possible that the mutant RNAP efficiently recognized the σ^A-dependent promoters, resulting in marked activation of the NTD biosynthesis pathway. On the other hand, assessment of *Streptomyces mauvecolor* 631689 demonstrated that two *rpoB* mutants (H437D or H437L) produced a family of novel antibiotics, the piperidamycins. In this case, the activation of silent genes was attributed, at least in part, to the increased affinity of mutant RNAP for the silent gene promoters (18). The observed upregulation of cryptic genes by *rpoB* mutations in actinomycetes (Fig. 2) could be accounted for, at least in part, in this way. In contrast, the downregulation observed in several cryptic genes (Fig. 2) may be accounted for by the decreased affinity of mutant RNAP for the promoters.

The amino acid alteration at position H437 (corresponding to H406, H482 and H526 in *Thermus thermophilus*, *B. subtilis*, and *Escherichia coli*, respectively) was often effective in activating the cryptic pathways (Table 2). The mutation at position H437 has been shown to circumvent the detrimental effects of the *relA* and *afsB* mutations (in *S. coelicolor*) and the *relC* mutation (in *S. lividans*) on actinorhodin production, perhaps by mimicking the ppGpp-bound form of RNAP (40–43). Taken together, ppGpp likely participates significantly even in the activation of cryptic gene clusters as well as highly expressed, well-known, secondary metabolite gene clusters. Precocious overexpression of cryptic gene clusters and cell lysis observed at late growth phase of *S. griseus* with the *rpoB* H437Y mutation (see Fig. S2 in the supplemental material) can be explained by its unique spectrum of effects in modulating the transcription of each gene. In fact, the *rpoB* S444F mutation (corresponding to S442F in this study) of *S. erythraea* markedly alters the transcriptional profile of this organism (24). The defective effects of the *rpoB* H437Y mutation on sporulation and competence were also reported in *B. subtilis* (44).

In addition to marked activation of cryptic genes, it should be emphasized that the *rpoB* mutations actually rendered cells active in producing a number of metabolites, which were not detected in the wild-type strain (Fig. 3A). These results demonstrated that introducing the *rpoB* mutations not only enhanced expression of the cryptic genes at the transcriptional level but could be effective in discovering novel secondary metabolites. It is also notable that differential effects of *rpoB* mutation were observed at both the gene transcription level (Fig. 2A) and the metabolite level (Fig. 4).The discovery of a new actinorhodin-related compound in the *S. coelicolor rpoB* mutant encourages us to utilize the *rpoB* mutation approach for drug discovery. The proposed structure of the new actinorhodin-related compound based on its molecular size most closely resembles ε-actinorhodin (see Fig. S5B in the supplemental material), and these two actinorhodin-related compounds are major metabolites in the *rpoB* mutant KO-1130 (see Fig. S5A), possibly reflecting a close relationship in biosynthesis.

Flavonoids, exuded by plant cells, are abundant in soil, especially in the rhizosphere. Certain flavonoids possess antibacterial activity and, interestingly, induce the expression of certain bacterial genes (45). The flavonoids examined, however, had no significant effects on the expression of *S. griseus* cryptic genes (Table 4). Similarly, the expression was not significantly affected by heavy metals, such as Cu, Zn, Co, Mn, and Ga. Thus, the effect of rare earth elements on activation should be highlighted. The rare earth elements have recently been shown to be involved in the overproduction of antibiotics or enzymes (46, 47). The effects of scandium were exerted at the level of transcription of pathway-specific regulatory genes, as demonstrated by marked upregulation of *actII*-ORF4 in *S. coelicolor* (47). Notably, rare earth elements were effective not only in activating *actII*-ORF4 but also in activating silent or poorly expressed secondary metabolite biosynthetic genes (27). As rare earth elements are distributed ubiquitously in soil, it is possible that microorganisms have acquired the ability to respond to low levels of these elements over the course of their long evolutionary history, possibly as a means of adapting their physiology to prevailing conditions.

In addition to our approach, other methods have been developed to activate silent biosynthetic pathways in *Streptomyces*, including manipulation of nucleoid structure; the addition of *N*-acetylglucosamine to the medium or deletion of the *dasR* gene, which encodes an *N*-acetylglucosamine-responsive regulatory protein; the constitutive overexpression of a pathway-specific LAL regulatory gene; metabolic remodeling; and cell-to-cell interaction (reviewed by Ochi and Hosaka [13]). These are all characterized by applicability to a wide range of actinomycetes and potential scalability to high-throughput studies. Myxobacterial genomes have been shown to encode many genes involved in

the synthesis of secondary metabolites (e.g., 8.6% of the *Myxococcus xanthus* genome), suggesting the possibility of discovering clinically relevant natural products (48). Hence, activation or enhancement of cryptic genes of this microbial group by introducing the *rpoB* mutation or by other approaches is of particular interest. Apart from the technology, it is important to envisage why cryptic genes are silent under laboratory fermentation conditions. Understanding the mechanism(s) underlying the silencing of cryptic genes would facilitate the full utilization of the microbial gene clusters for secondary metabolism.

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