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Dramatic Activation of Antibiotic Production in *Streptomyces coelicolor* by Cumulative Drug Resistance Mutations[▽]†

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We recently described a new method to activate antibiotic production in bacteria by introducing a mutation conferring resistance to a drug such as streptomycin, rifampin, paromomycin, or gentamicin. This method, however, enhanced antibiotic production by only up to an order of magnitude. Working with *Streptomyces coelicolor* A3(2), we established a method for the dramatic activation of antibiotic production by the sequential introduction of multiple drug resistance mutations. Septuple and octuple mutants, C7 and C8, thus obtained by screening for resistance to seven or eight drugs, produced huge amounts (1.63 g/liter) of the polyketide antibiotic actinorhodin, 180-fold higher than the level produced by the wild type. This dramatic overproduction was due to the acquisition of mutant ribosomes, with aberrant protein and ppGpp synthesis activity, as demonstrated by in vitro protein synthesis assays and by the abolition of antibiotic overproduction with *relA* disruption. This new approach, called "ribosome engineering," requires less time, cost, and labor than other methods and may be widely utilized for bacterial strain improvement.

Strain improvement is important in applied microbiological research, especially in the production of clinically important antibiotics as well as antibiotics important in veterinary medicine and agriculture. Current methods of antibiotic production, ranging from classical random approaches to metabolic engineering, are either costly or labor-intensive. We recently described a new method to increase antibiotic production in bacteria by modulating ribosomal components (ribosomal proteins or rRNA), i.e., by the introduction of mutations conferring drug resistance, since many antibiotics target the ribosome (13, 29, 31). This new approach, called "ribosome engineering" (28), has several advantages. These advantages include 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. Thus, this method requires no induced mutagenesis. This approach has been used to enhance the production of salinomycin in an industrial strain of *Streptomyces albus* (33); to activate the synthesis of dormant antibiotics (15, 19); to improve chemical tolerance in *Pseudomonas putida* (12), a valuable bacterium for waste processing; and to enhance the synthesis of enzymes such as α-amylase in *Bacillus subtilis* (22). Interestingly, the introduction of several drug resistance mutations had a cumulative effect on antibiotic production. This was shown by the sequential introduction of three drug resistance mutations into *Streptomyces coelicolor* A3(2) (*str*, *gen*, and *rif*, which confer resistance to streptomycin [Sm], gentamicin [Gen], and rifampin [Rif], respectively) and three rounds of selection, with the resulting triple mutant, SGR, showing hierarchical increments of antibiotic production (14).

S. coelicolor A3(2), the genetically best-characterized strain of *Streptomyces*, produces at least four distinct classes of antibiotics (21), including the blue-pigmented polyketide antibiotic actinorhodin (Act), thus providing an easily tractable system for the methodological study of strain improvement. Based on our previous results using single mutations or multiple mutations, we demonstrate here the efficacy of septuple and octuple drug resistance mutations for the dramatic activation of antibiotic production. The mechanisms underlying this remarkable activation were also studied.

MATERIALS AND METHODS

Drugs and chemicals. Geneticin, paromomycin (Par), fusidic acid (FA), lincomycin (Lin), and thiostrepton (Tsp) were purchased from Sigma; Sm was purchased from Nacalai Tesque, Inc. (Japan); and Gen, Rif, tetracycline (Tet), and hygromycin were purchased from Wako Pure Chemicals (Japan). The standards guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) were prepared enzymatically in our laboratory, each with a purity of >95%.

Bacterial strains and preparation of multiply drug-resistant mutants. *S. coelicolor* A3(2) wild-type strain 1147, triple mutant strain SGR (14), and its multiply drug-resistant derivatives are listed in Table 1. All of the mutations arose spontaneously, and no induced mutagenesis was required. For each experiment, strains were inoculated onto GYM (31) or SFM (21) plates and incubated for approximately 7 to 10 days for sporulation. Fresh spore suspensions were inoculated into 100 ml medium in a 500-ml flask and incubated on a rotary shaker (200 rpm) at 30°C except where indicated.

Triple mutant strain SGR (14) was used as the starter strain to select for multiple-drug-resistant mutants, with general screening procedures performed as described previously (28). The procedure used to prepare multiply drug-resistant mutants is illustrated in Fig. 1A. Spore suspensions (usually $>10^9$ spores) were spread and incubated for 7 days. Antibiotic-resistant clones were first screened for the production of Act on plate cultures, with candidates having the deepest blue color being assessed for Act production assays using liquid media. If necessary, mutations were mapped by DNA sequencing. In each step, one or two colonies with the highest Act production were used for the next round of screening. SGR spore suspensions were spread onto GYM plates containing various concentrations of Par, usually approximately 3- to 30-fold of the parental strain's MIC to select for Par resistance, followed by similar steps of screening for

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TABLE 1. *S. coelicolor* A3(2) and its drug-resistant derivatives used in this study

Strain	Genotype or description[f]	Mutation introduced at each step[a]	Reference or source
1147	Prototrophic wild type	—[a]	21
K88E	*str*	K88E in ribosomal protein S12	8
SG	*str gen*	ND	14
SGR	*str gen rif*	H437Y in RNA polymerase β-subunit	14
SGRP	*str gen rif par*	Insertion of a glycine residue at position 92 of S12	This study
C5	*str gen rif par gnt*	—[b]	This study
C6	*str gen rif par gnt fus*	—[c]	This study
C7	*str gen rif par gnt fus tsp*	—[d]	This study
C8	*str gen rif par gnt fus tsp lin*	—[e]	This study
C8relA2	*str gen rif par gnt fus tsp lin relA*	Insertional disruption of *relA* gene by hygromycin resistance cassette (*hyg*)	This study

[a] No mutation; wild type.
[b] Mutation not determined.
[c] Mutation not detected in elongation factor G.
[d] Mutation not detected in ribosomal protein L11.
[e] Mutation not detected in ribosomal proteins S7, L14, and L15.
[f] *str*, *gen*, *rif*, *par*, *gnt*, *fus*, *tsp*, and *lin* confer resistance to streptomycin, gentamicin, rifampin, paromomycin, Geneticin, fusidic acid, thiostrepton, and lincomycin, respectively.

resistance to Geneticin, FA, Tsp, and Lin, finally yielding octuple mutants with resistance to eight different drugs.

Antibiotic production assay. GYM, R2YE (21), R5 (21), and R3 (31) media were used to screen for the production of the blue antibiotic Act on agar plates by directly assessing the density of the blue color. For Act production in liquid media, culture samples (0.5 ml) were mixed with equal volumes of 2 M KOH, vortexed, and centrifuged at 3,000 × *g* for 5 min. The Act concentration in the supernatants was determined by measuring the absorbance at 640 nm (optical density at 640 nm [OD_{640}]; ε = 25,320 for the pure compound) (21). Red in liquid GYM medium and calcium-dependent antibiotic (CDA) on plates were assayed as described previously (21). For antibiotic production, three flasks were always used for each strain, with production confirmed by at least two separate experiments.

Determination of MICs. To determine MICs, spore solutions were dotted onto GYM plates containing various concentrations of a drug and incubated at 30°C for 48 h, with the minimum drug concentration able to fully inhibit growth defined as the MIC.

Mutation analysis. Primers used to amplify the candidate DNA fragments are listed in Table S1 in the supplemental material. PCR amplification was carried out with ExTaq (Takara). Purified PCR products were directly sequenced with BigDye Terminator cycle sequencing kits (Perkin-Elmer Applied Biosystems, Foster City, CA). The sequence data were aligned using the GENETIX program (Software Development Co., Tokyo, Japan).

Disruption of *relA*. *S. coelicolor* A3(2) is characterized by high-frequency conjugation due to the presence of the sex factor plasmid SCP1 (21). To knock out the *relA* gene in the octuple mutant C8, conjugation was performed using the *relA* null mutant M570 (5), in which most of the *relA* gene (corresponding to amino acid residues 167 to 683 of 847 residues) was replaced by a hygromycin resistance gene (*hyg*). Strains C8 and M570 were mix cultured on a R2YE plate, and the spores that formed after 5 days were spread onto a selection plate containing 5 μg/ml of hygromycin and all eight drugs. The conjugant (C8relA2) thus obtained was assayed by PCR for the absence of the internal segment of *relA* and the presence of *hyg*.

Analysis of gene transcription. Total RNAs were purified from cells grown on GYM plates covered with cellophane for the indicated times using Isogen reagent (Nippon Gene) according to the manufacturer's protocol. After treatment with RNase-free DNase I (amplification grade; Invitrogen), 1 μg of each of the total RNAs was used as a template for reverse transcription (RT) (20 μl) with a ThermScript RT-PCR kit (Invitrogen). Primers used for RT-PCR are listed in Table S1 in the supplemental material. The amount of RT products used as a PCR template and numbers of PCR cycles were optimized for each gene. In a 50-μl PCR mixture, 1 and 2 μl of reverse transcript products were used for *act*II-ORF4 and *hrdB* (encoding the major sigma factor), respectively, which were amplified with 26 cycles, and 4 μl was used for *relA* for 28 cycles.

Real-time quantitative PCR (qPCR) analysis of gene transcription was conducted using the 7300 real-time PCR system and Sybr green PCR master mix (Applied Biosystems) as described previously by Kasai et al. (20), except that random hexamers included in the ThermScript RT-PCR kit (Invitrogen) were used in the RT reaction and except that the annealing temperature of qPCR was 60°C. The transcription of *hrdB*, a gene encoding the principal sigma factor of RNA polymerase, was used as the internal control. Each transcriptional assay was normalized to the corresponding transcriptional level of *hrdB*. Primers used for real-time qPCR are listed in Table S1 in the supplemental material.

Preparation of ribosomes and the S-150 fraction. Ribosomes and the S-150 fraction (i.e., the supernatant following centrifugation at 150,000 × *g* for 3 h) were prepared from cells harvested at various growth phases in YEME medium (21), as described previously (11), except that cells were first washed twice with a high-salt buffer containing 1 M (instead of 30 mM) ammonium acetate before washing with the standard buffer and except that 2 mM (instead of 1 mM) phenylmethylsulfonyl fluoride was included in all buffers.

In vitro protein synthesis. Cell-free synthesis of green fluorescent protein (GFP) was performed as described previously (11). In brief, 20 A_{260} units/ml of ribosome or 0.5 mg/ml of the S-150 fraction was incubated at 30°C for 15 min in a 100-μl total volume of a mixture containing 1.2 mM ATP, 0.8 mM GTP, 0.64 mM cyclic AMP, 15 μg *Escherichia coli* total tRNAs, and 0.4 mM each of the 20 natural L-amino acids. GFP synthesis was initiated by the addition of 100 μg *gfp* mRNA, which had been synthesized in vitro from a plasmid *gfp* gene using T7 RNA polymerase (Takara). Aliquots (10 μl) were withdrawn every 30 min, electrophoresed on native 10% polyacrylamide gels, and subjected to fluorescence analysis using a FluoroImager (Molecular Dynamics).

Assay of ppGpp. Intracellular ppGpp was extracted from cells cultured on GYM and on modified R5 (16) plates covered with cellophane with 1 M formic acid and assayed by high-performance liquid chromatography (27). For nutritional shift down, exponentially growing cells in chemically defined (CD) medium (27) supplemented with 3% Casamino Acids (vitamin free; Difco) were rapidly transferred into fresh CD medium without Casamino Acids. Cells were harvested 0, 15, 30, and 60 min after cell transfer for the extraction of ppGpp; pppGpp was not detectable in these samples.

RESULTS AND DISCUSSION

Construction of multiply drug-resistant mutants. Starting with triple mutant strain SGR (14), we screened for Par resistance by spreading SGR spores onto GYM plates containing various concentrations of Par, as illustrated in Fig. 1A. About 20% of the colonies that developed within 7 days due to spontaneous mutations showed increased Act production compared with that of parental strain SGR. Since we previously found that a Par resistance mutation (P91S) in ribosomal protein S12 effectively resulted in Act overproduction (29), we sequenced the S12-encoding gene, *rpsL*, in SGR and dozens of Act-overproducing colonies. As expected, we found that in addition to the *rpsL*-encoded mutation K88E present in the SGR mutant, all colonies tested possessed a second amino acid substitution in S12, including the P91S mutation. To our surprise, we found that one colony contained a novel Par resistance mutation

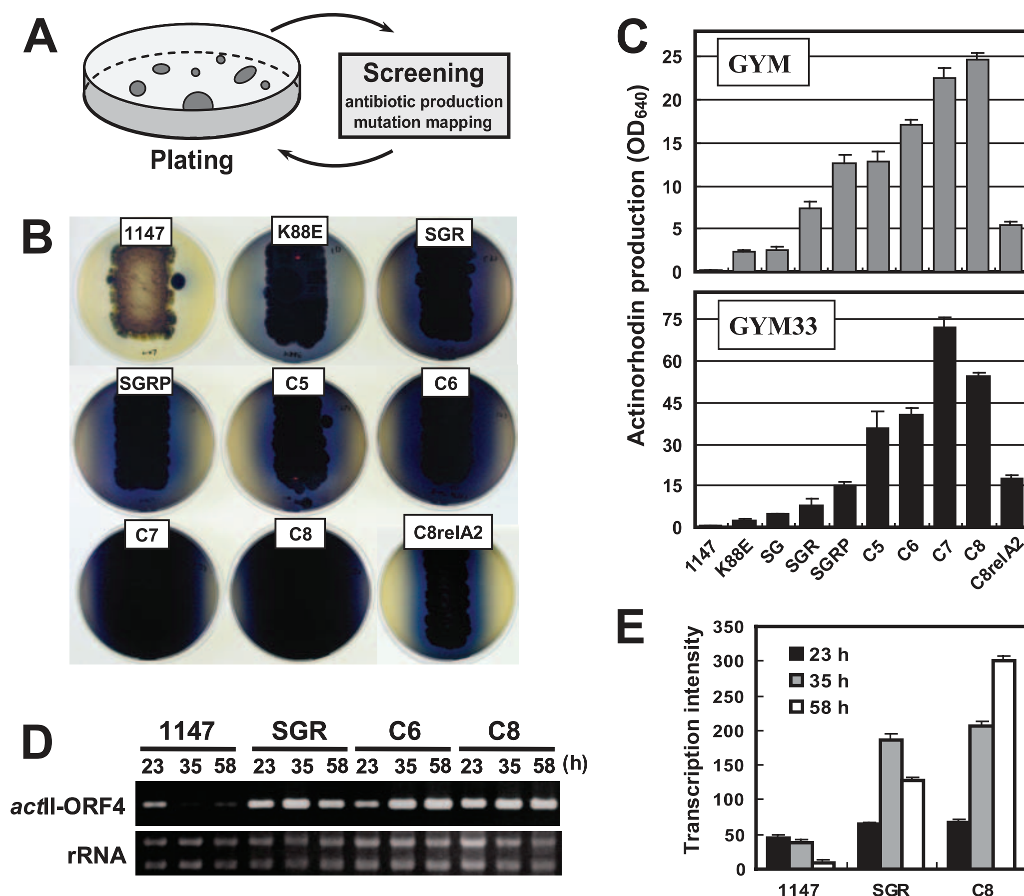

FIG. 1. Act production and *act*II-ORF4 transcription in drug-resistant mutants. (A) Illustration of the procedure used for the sequential introduction of drug resistance mutations to generate multiple-drug-resistant mutants. (B) Act production on agar plates. Strains were inoculated onto GYM plates, which were incubated for 8 days. The reverse sides of the plates are shown to illustrate the blue antibiotic Act. (C) Act production in liquid culture. Strains were inoculated into liquid GYM or GYM33 medium and incubated on a rotary shaker (200 rpm) for 8 days. Act production by each strain was measured as the maximum OD_{640} value. (D) Transcriptional analysis of *act*II-ORF4 by RT-PCR. Total RNA preparation and RT-PCR were performed as described in Materials and Methods. Eight microliters of each PCR product was loaded for electrophoresis. The gel profile of rRNA (4 μg per lane) is presented as a reference. (E) Transcriptional analysis of *act*II-ORF4 by real-time qPCR. Total RNA preparation and real-time qPCR were performed as described in Materials and Methods. Each transcriptional assay was normalized to that of *hrdB*. The error bars indicate the standard deviations of the means of triplicate samples.

consisting of an inserted glycine residue (G, encoded by ggg) at position 92 of S12 (92::G), although it appeared at a relatively low frequency. More interestingly, this new insertion mutation plus K88E rendered cells able to produce even more Act than P91S combined with K88E. The introduction of 92::G (and P91S) (not shown) increased the sensitivities of mutants to many antibiotics, including Geneticin, FA, Lin, and Tet (Table 2). Consequently, a quadruple mutant, SGRP, containing 92::G was used for further selection.

Subsequent sequential screenings were performed to isolate

TABLE 2. Levels of resistance of *S. coelicolor* A3(2) strains to various drugs

Strain	MIC (μg/ml)[a]								
	Sm	Gen	Rif	Par	Geneticin	FA	Tsp	Lin	Tet
1147	1	0.1	10	0.1	0.5	90	1	50	10
K88E	100	0.1	30	0.3	0.8	90	1	50	10
SG	100	0.3	30	0.3	0.8	90	1	50	10
SGR	100	0.3	300	0.3	0.5	70	1	50	10
SGRP	100	0.3	300	5	0.3	30	1	8	5
C5	100	0.4	300	5	0.5	30	0.5	10	5
C6	100	0.4	300	10	0.8	70	1	8	5
C7	100	0.3	300	10	0.3	70	50	8	2
C8	100	0.3	300	10	0.3	90	50	200	3

[a] The MICs were determined after incubation on GYM plate at 30°C for 48 h.

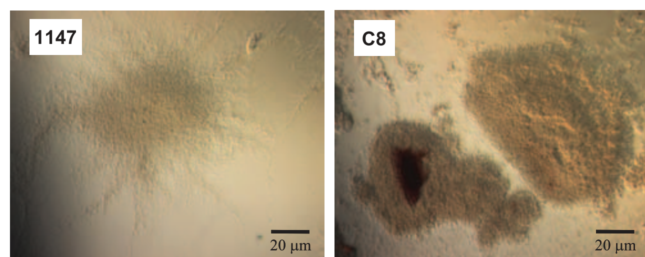

FIG. 2. Morphological appearance of wild-type strain 1147 and octuple mutant strain C8 during exponential growth phase in liquid R2YE medium. Bars represent 20 μm.

strains that were resistant to Geneticin, FA, Tsp, and Lin, generating quintuple, sextuple, septuple, and octuple mutants (C5, C6, C7, and C8, respectively) (Table 1). During these screenings, we found that as many as 5% to 15% of the mutant strains produced more Act than the corresponding parental strain. The period required for all screenings was 8 months. In general, as the number of mutations increased, the growth rate slowed (see Fig. 3A), a finding consistent with the opinion that drug resistance was often obtained at the cost of growth fitness (1).

Octuple mutant strain C8 was somewhat temperature sensitive; wild-type strain 1147 grew at 40°C, but C8 did not grow at 39°C. In addition, C8 exhibited extremely slow growth in a chemically defined medium. In liquid culture, mutant strain C8 showed a tight "core" pellet with short-fragmented mycelia, whereas the wild type (1147) possessed a core surrounded by many naturally stretching, branched mycelia (Fig. 2). Importantly, mutant strain C8 was genetically stable; no obvious changes in morphology and Act productivity were found even after 16 sequential inoculations onto GYM agar plates.

Antibiotic overproduction and mutation analysis. The relationship between Act production and the number of mutations is shown in Fig. 1B and C. We found that C7 produced 0.51 g/liter and that C8 produced 0.55 g/liter Act, 88- and 95-fold higher, respectively, than the 5.8 mg/liter produced by wild-type 1147 in liquid GYM medium. It was noteworthy that low temperatures could further enhance Act production by these multiply drug-resistant mutants. When strains were incubated at 27°C, C7 produced 0.63 g/liter Act, and C8 produced 0.90 g/liter Act, or 24% and 64% higher, respectively, than at 30°C. Increased Act production was more pronounced when mutants were cultivated in a dense medium, GYM33 (GYM medium supplemented with 3% starch and 3% soybean powder). In this medium, C7 and C8 produced 1.63 g/liter and 1.22 g/liter Act, respectively, or 180- and 136-fold more than the 9 mg/liter produced by wild-type strain 1147 (Fig. 1C).

Transcriptional analysis by RT-PCR showed that the expression of the *act*II-ORF4 gene, encoding the positive regulator of Act biosynthesis (7), was greatly enhanced in drug-resistant mutants, especially in the late phase of mutants (C6 and C8) (Fig. 1D). This was confirmed by real-time qPCR, in which the expression level of *act*II-ORF4 in the C8 mutant was sevenfold higher than that in wild-type strain 1147 (Fig. 1E). These results indicate that drug resistance mutations exerted their effects on antibiotic production at the transcriptional level. In addition to Act, these multiply drug-resistant mutants showed increased productivity of undecylprodigiosin (red) and CDA (data not shown), although the observed increase (20- to 30-fold) was not as dramatic as that of Act.

Structural studies on the mechanism of action of antibiotics have shown that many of these agents target ribosomal components (35, 36). For example, most FA resistance mutations are clustered in the *fusA* gene, which encodes elongation factor G (3); Tsp resistance is often due to mutations in or a deletion of the ribosomal protein L11 (27, 30), and Lin resistance is frequently due to mutations in the ribosomal proteins S7, L14, and L15 (17). Octuple mutant strain C8, however, did not have mutations in the *fusA* gene, the L11-encoding gene *rplK*, and the genes encoding S7, L14, and L15. Apparently, new types of drug resistance mutations tend to appear in these "unnatural ribosomes" that contain multiple drug resistance mutations.

Mutant ribosomes sustain a high level of protein synthesis even at the late growth phase. Although some mutations were not identified, it is likely that ribosomal properties and concomitant protein translation were modified largely by drug resistance mutations, because all drugs used in this study, except rifampin, target ribosomal components. We therefore assessed the in vitro protein synthesis activities of the mutants using nascent GFP mRNA as a template. Ribosomal and S-150 fractions were prepared from wild-type 1147, triple mutant strain SGR, and octuple mutant strain C8 cells grown in YEME medium to mid-exponential phase (E), early stationary phase (S2), and late stationary phase (S4) (Fig. 3A). GFP was abundantly synthesized by ribosomes prepared from cells of all strains in the E phase. Ribosomes prepared from wild-type cells in S2 synthesized much less GFP, and ribosomes prepared from wild-type cells in S4 lost their ability to synthesize protein. By contrast, ribosomes prepared from drug-resistant mutant strains SGR and C8 in both S2 and S4 showed high levels of protein synthesis activity (Fig. 3B). Of note, ribosomes prepared from the octuple mutant C8 during exponential and stationary phases maintained relatively constant levels of protein synthesis activity, decreasing only about 25% upon entering stationary phase (Fig. 3B). Furthermore, ribosomes prepared from cells at an extremely late growth phase (S5) (Fig. 3A) had over 60% protein synthesis activity compared with ribosomes from E-phase cells (data not shown). Thus, the ability of late-growth-phase cells to sustain a high level of protein synthesis activity appears to be a key feature of drug-resistant mutants, as we demonstrated previously (11), an activity especially important for the expression of stationary-phase-specific genes, including those for secondary metabolism.

Mutant cells acquire an increased ability to accumulate ppGpp. Bacterial cells exert "stringent control" over a wide variety of genes and enzymes when they encounter adverse environmental conditions, such as the limited availability of an essential nutrient. ppGpp is a key mediator of this response and is therefore called a bacterial alarmone (4). Experiments using knockout mutants of genes necessary for ppGpp synthesis or forcing ppGpp synthesis under nutrient-sufficient conditions have indicated that ppGpp plays a pivotal role in the onset of antibiotic production in bacteria, including *Streptomyces* spp. (5, 6a, 9, 26, 27) and *B. subtilis* (18).

Strikingly, when grown on GYM agar, the octuple mutant C8 had a much higher intracellular level of ppGpp (4.5 to 13

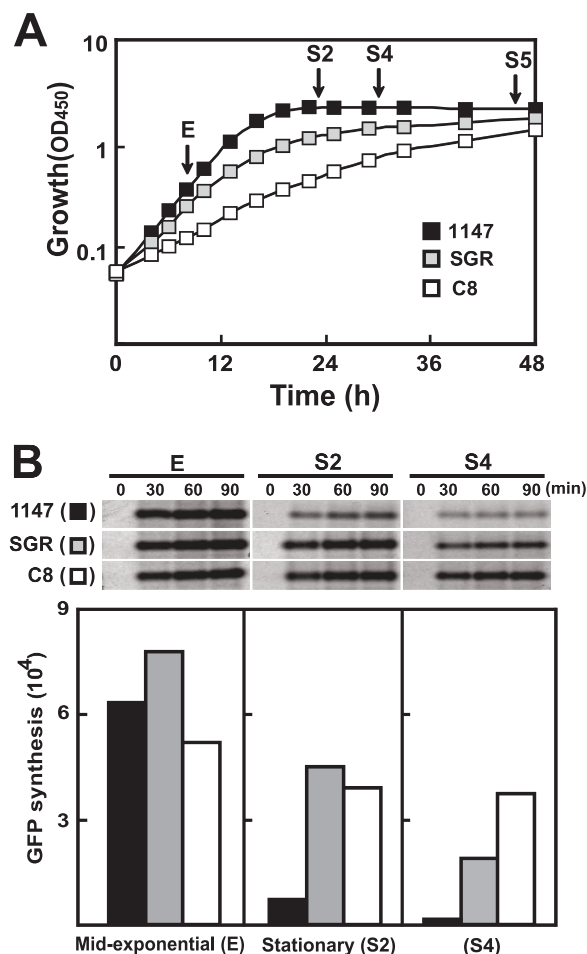

FIG. 3. Growth patterns and in vitro protein synthesis activities of wild-type strain 1147, triple mutant strain SGR, and octuple mutant strain C8. (A) Growth profiles. Spores were inoculated into liquid YEME medium, with absorbance at 450 nm (OD_{450}) monitored for the growth curve. An OD_{450} of 0.06 was defined as the zero time point approximately 16 to 24 h after inoculation. Arrows indicate the time points at which cells were harvested for preparation of ribosomes and the S-150 fraction. (B) In vitro GFP synthesis was assayed using ribosomes from 1147 (■), SGR (▩), and C8 (□), with nascent *gfp* mRNA as the template. Top, fluorographs. Bottom, fluorescence intensities of bands after 90 min of reaction, as determined by scanning the fluorographs.

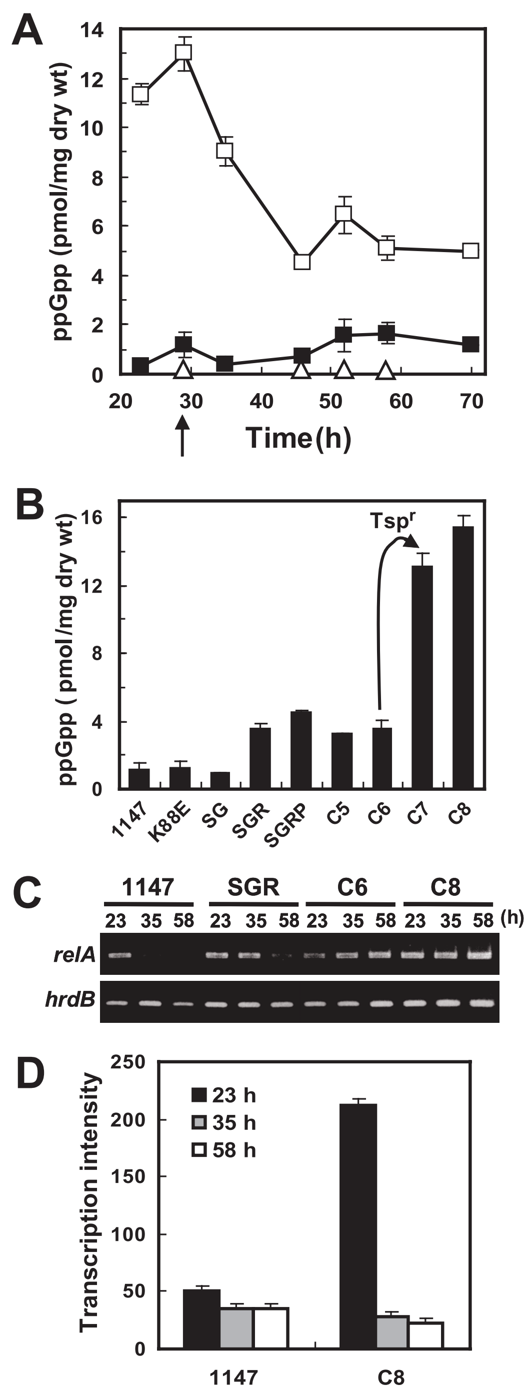

FIG. 4. Accumulation of intracellular ppGpp and transcriptional analysis of the *relA* gene in drug-resistant mutants. (A) Intracellular ppGpp levels of wild-type strain 1147, octuple mutant strain C8, and *relA* deletion mutant strain C8relA2 during growth on GYM plates. Cells were harvested at the indicated time points for ppGpp extraction. ■, 1147; □, octuple mutant strain C8; △, *relA* deletion mutant strain C8relA2. (B) Intracellular ppGpp levels of each drug-resistant mutant at 29 h (corresponding to the arrow in A). The increase in the ppGpp level upon the introduction of Tsp resistance is indicated by an arrow. (C) Transcriptional analysis of *relA* in drug-resistant mutants by RT-PCR using the total RNA samples as described in the legend of Fig. 1D. Transcription of *hrdB* was utilized as an internal control. (D) Transcriptional analysis of *relA* by real-time qPCR. Total RNA preparation and real-time qPCR were performed as described in Materials and Methods. Each transcriptional assay was normalized to that of *hrdB*. The error bars indicate the standard deviations of the means of triplicate or more samples.

pmol/mg [dry weight]) than wild-type strain 1147 (0.3 to 1.6 pmol/mg [dry weight]) (Fig. 4A). The high level of ppGpp in C8 was especially pronounced during the early growth phase (20 to 30 h), when its ppGpp level was 10- to 30-fold higher than that in the wild type. Similar results were obtained in liquid culture (see Fig. S1 in the supplemental material) and on modified R5 plates (data not shown). The ability to accumulate a high level of ppGpp was conferred mainly upon the introduction of Tsp resistance, as measured by intracellular ppGpp levels of each drug-resistant mutant at 29 h (Fig. 4B). To clearly demonstrate the intrinsic role of mutant ribosomes, the ability of the mutant to synthesize ppGpp was analyzed in shift-down experiments. In both wild-type and mutant strains, ppGpp synthesis was activated immediately after the depletion of amino acids, peaking 15 min after nutritional shift down. As

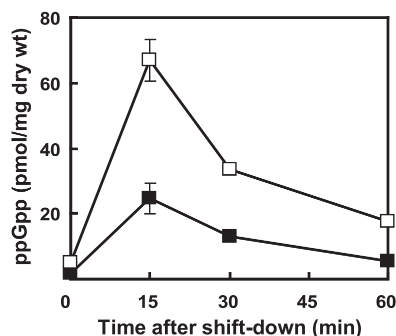

FIG. 5. Intracellular ppGpp accumulation after nutritional shift down. After growth to mid-exponential phase in CD medium plus 3% Casamino Acids, cells were rapidly transferred into fresh CD medium without Casamino Acids, and ppGpp was extracted with 1 M formic acid at the indicated time points. The error bars indicate the standard deviations of the means of triplicate samples. ■, wild-type strain 1147; □, octuple mutant strain C8.

expected, we found that C8 synthesized threefold more ppGpp than did wild-type strain 1147 (Fig. 5), indicating that C8 mutant ribosomes synthesized and accumulated higher levels of ppGpp.

Although the detailed molecular mechanisms underlying these phenomena are at present not known, it is important that the expression of the *relA* gene, which encodes ppGpp synthetase, was elevated remarkably at the transcriptional level in the mutants, as shown by RT-PCR analysis (Fig. 4C) and as confirmed by real-time qPCR analysis, which displayed a 4.4-fold-elevated level of transcription of the *relA* gene in mutant strain C8 (Fig. 4D). It was also confirmed that these multiply drug-resistant mutants have no mutations in the *relA* gene. Several kinds of drug resistance mutations (including *rif* and *fus* mutations) were previously reported to disturb ppGpp synthesis (23, 24) but show a reduction in the ppGpp level. Our finding is therefore the first to demonstrate the existence of mutations (especially a *tsp* mutation) that cause an increase in ppGpp levels.

***relA* disruption abolishes a mutant's antibiotic overproduction.** To confirm the important role of ppGpp in the dramatic activation of Act production in multiply drug-resistant mutants, the *relA* gene was knocked out in octuple mutant strain C8 by conjugation with *relA* null mutant strain M570 (5), which is entirely deficient in ppGpp synthesis. This conjugant, C8relA2, showed a complete inability to accumulate ppGpp throughout all growth phases (Fig. 4A) as well as a >60% decrease in Act production (Fig. 1B and C), indicating the critical role of elevated ppGpp levels in the antibiotic overproduction observed in mutant strain C8. The impaired growth of mutant strain C8 was restored only partially by introducing the *relA* mutation (data not shown).

Recent analyses of the ppGpp system in plant cells (6, 32, 34) and X-ray structural analysis of the RNA polymerase-ppGpp complex (2) have enhanced our understanding of the physiological significance of the stringent control mediated by ppGpp. Analysis of the effect of ppGpp accumulation on changes in the *S. coelicolor* A3(2) transcriptome suggested a global role for ppGpp in cellular function (10). The induction of ppGpp synthesis activated the transcription of genes for Act and CDA biosynthesis, a finding in good agreement with the results shown here.

Concluding remarks. We have developed a more rapid and cost-effective and less labor-intensive method (i.e., ribosome engineering) for the dramatic activation of antibiotic production by constructing multiply drug-resistant mutants. Our results demonstrate that this dramatic overproduction of antibiotics was due to mutant ribosomes with aberrant protein and ppGpp synthesis activities. These findings indicate that ribosomes can be good targets for strain improvement in bacteria, although it is still unclear why ribosomal mutations markedly enhance *relA* transcription (Fig. 4C and D). We reported similar results for *metK* (coding for *S*-adenosylmethionine synthetase), the expression of which was activated more than 30-fold by an *rsmG* mutation in *S. coelicolor*, which results in the failure to methylate 16S rRNA at position G518 (25). Clarification of these peculiar phenomena at the molecular level may open new horizons for the study of still-unknown ribosomal functions. Although in the present study, we used mainly three strains (1147, SGR, and C8) as representatives to be characterized in some detail, more convincing evidence should come from a thorough analysis of each successive step in mutagenesis, since ribosomal alterations and altered ppGpp synthesis abilities do not necessarily occur together or occur in each successive mutant strain.

The present findings are in agreement with our proposal (11, 28) that the cell's capacity to synthesize protein at a late growth phase is crucial for accelerating the initiation of onset of secondary metabolism and for the production of abundant biosynthetic enzymes. The principal regulator of Act production in *S. coelicolor* appears to be the availability of the pathway-specific transcriptional regulatory protein ActII-ORF4, a threshold concentration of which is required for the efficient transcription of its cognate biosynthetic structural genes (7). Although we do not yet know how the drug resistance mutations mediated preferential gene transcription work (Fig. 1D and E), it is conceivable that the expression of pathway-specific regulatory genes (e.g., *act*II-ORF4 and *redD*) is governed by higher-order regulatory proteins and that the expression of the latter presumptive regulatory proteins may be significantly affected under conditions associated with enhanced protein synthesis during the stationary phase in the mutants.

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