

Mutations in *rsmG*, Encoding a 16S rRNA Methyltransferase, Result in Low-Level Streptomycin Resistance and Antibiotic Overproduction in *Streptomyces coelicolor* A3(2)^{∇†‡}

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Certain *str* mutations that confer high- or low-level streptomycin resistance result in the overproduction of antibiotics by *Streptomyces* spp. The *str* mutations that confer the high-level resistance occur within *rpsL*, which encodes the ribosomal protein S12, while those that cause low-level resistance are not as well known. We have used comparative genome sequencing to determine that low-level resistance is caused by mutations of *rsmG*, which encodes an *S*-adenosylmethionine (SAM)-dependent 16S rRNA methyltransferase containing a SAM binding motif. Deletion of *rsmG* from wild-type *Streptomyces coelicolor* resulted in the acquisition of streptomycin resistance and the overproduction of the antibiotic actinorhodin. Introduction of wild-type *rsmG* into the deletion mutant completely abrogated the effects of the *rsmG* deletion, confirming that *rsmG* mutation underlies the observed phenotype. Consistent with earlier work using a spontaneous *rsmG* mutant, the strain carrying $\Delta rsmG$ exhibited increased SAM synthetase activity, which mediated the overproduction of antibiotic. Moreover, high-performance liquid chromatography analysis showed that the $\Delta rsmG$ mutant lacked a 7-methylguanosine modification in the 16S rRNA (possibly at position G518, which corresponds to G527 of *Escherichia coli*). Like certain *rpsL* mutants, the $\Delta rsmG$ mutant exhibited enhanced protein synthetic activity during the late growth phase. Unlike *rpsL* mutants, however, the $\Delta rsmG$ mutant showed neither greater stability of the 70S ribosomal complex nor increased expression of ribosome recycling factor, suggesting that the mechanism underlying increased protein synthesis differs in the *rsmG* and the *rpsL* mutants. Finally, spontaneous *rsmG* mutations arose at a 1,000-fold-higher frequency than *rpsL* mutations. These findings provide new insight into the role of rRNA modification in activating secondary metabolism in *Streptomyces*.

One of the most intriguing challenges in biology is the elucidation of the mechanisms whereby cells switch from primary to secondary metabolism in response to extracellular nutritional conditions. Among prokaryotes, *Streptomyces* spp. provide a tractable experimental system for studying such mechanisms due to their display of a wide range of adaptations to extreme nutrient limitations, including the production and secretion of antibiotics and enzymes and the formation of aerial mycelium and spores (4, 9). *Streptomyces coelicolor* A3(2) is the best-characterized strain in this genus and has been used to study mechanisms regulating antibiotic production (7). This strain produces several chemically diverse antibiotics, including the red-pigmented tripyrrole undecylprodigiosin (Red) and the deep blue-pigmented polyketide actinorhodin (Act). We previously reported that the introduction of certain *str* mutations that confer streptomycin (Sm) resistance enhances Act production in both *S. coelicolor* A3(2) and *S. lividans* (12, 35). Moreover, an impaired ability to produce Act, resulting from certain developmental mutations (*relA*, *relC*, and *brgA*) in *S. coelicolor*, can be circumvented by introducing *str* mutations

(21, 23, 35). These *str* mutants have been classified into two types. Type I mutants carry a mutation within *rpsL*, which encodes the ribosomal protein S12, and exhibit high-level Sm resistance (MIC, >100 $\mu\text{g/ml}$), whereas type II mutants possess the wild-type *rpsL* gene and exhibit low-level Sm resistance (MIC, 5 to 10 $\mu\text{g/ml}$).

We previously demonstrated that *rpsL* mutant ribosomes carrying the K88E substitution in S12 (and K88R mutant ribosomes in *Streptomyces albus*) are more stable than wild-type ribosomes at low magnesium concentrations, indicating that this increase in stability could enhance protein synthesis (27, 38). We later found that increased expression of the translation factor ribosome recycling factor (RRF) also contributes to the enhanced protein synthesis observed during the late growth phase in the K88E *rpsL* mutant. This led us to conclude that both the greater stability of the 70S ribosome and the elevated levels of RRF caused by the K88E *rpsL* mutation are responsible for the enhanced protein synthesis seen during the late growth phase and that this underlies the observed overproduction of antibiotic in the K88E *rpsL* mutant (13).

In contrast to the pivotal role played by changes in ribosomal function in type I (*rpsL*) mutants, we found that a type II mutant, strain KO-179 (*str-19*), displays markedly enhanced expression of *S*-adenosylmethionine (SAM) synthetase (25). The importance of SAM synthetase activity in initiating Act production is supported by the results of RNase protection assays, which showed that overexpression of *metK* (encoding SAM synthetase) stimulates the expression of a positive regu-

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‡ This paper is dedicated to Keith F. Chater upon his retirement from the John Innes Institute.

† Supplemental material for this article may be found at <http://jba.asm.org/>.

[∇] Published ahead of print on 23 March 2007.

latory gene (*actIII*-ORF4) for the *act* gene cluster, and by the finding that the exogenous addition of SAM to the culture medium induces Act biosynthesis in wild-type cells (25). Similar results were reported for *S. lividans* (19). The molecular mechanism underlying the overexpression of SAM synthetase in type II *str* mutants, however, remains unknown.

Our ultimate aim is to develop "ribosome engineering" (22) as a rational approach to taking full advantage of bacterial capabilities. Toward that end, a detailed understanding of the mechanism(s) underlying the processes outlined above will increase our understanding of how enhanced production of Act occurs in *str* mutants. Sm was first shown to be a particularly potent drug against *Mycobacterium tuberculosis* in 1944 (34), and mutants resistant to Sm were reported as early as 1946 (20). However, the mechanism underlying low-level resistance to Sm (i.e., the type II mutation) has remained obscure. In the present work, we successfully identified a previously unknown mutation within *rsmG* (the gene encoding a 16S rRNA methyltransferase) that confers low-level Sm resistance. Moreover, further analysis of this mutation provided new insight into the role of rRNA modification in activating secondary metabolism in *Streptomyces*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Spores from *S. coelicolor* A3(2) strain 1147 (a prototrophic wild-type strain) and its derivatives were harvested from cultures grown on GYM agar medium (35). *Escherichia coli* strains DH5 α and GM2163 were used for routine DNA manipulation. R4 (35), R4C (R4 supplemented with 0.2% Casamino Acids), R5⁻ (14), and R5⁻ media without sucrose (R5MS) were used for Act production, with the latter assayed as described previously (18). Yeast extract malt extract (YEME) medium (18) and R5⁻ medium were used for studying in vitro protein synthesis and SAM synthetase activity, respectively. When necessary, agar medium was inoculated with about 10⁶ spores of *S. coelicolor*, the plates were covered with cellophane, and the cultures were grown at 30°C. Spontaneous low-level Sm-resistant mutants (*rsmG* mutants) were generated from wild-type strain 1147 (MIC, 1 μ g/ml) on GYM agar plates containing 3 μ g/ml Sm. Mutants exhibiting high-level Sm resistance (*psL* mutants) were obtained by plating spores on GYM agar medium containing 100 μ g/ml Sm. Serial dilutions of the cell suspension were also plated on media without Sm to determine the number of viable cells in the original suspension. To measure the frequency of resistant mutants, single colonies were isolated, and cells originating from each of about 10 to 20 clones were examined separately.

Isolation and manipulation of DNA and RT-PCR analysis. Plasmid isolation, restriction enzyme digestion, ligation, and transformation of *E. coli* and *Streptomyces* were performed as described previously (18, 33). PCR was performed using TaKaRa LA *Taq* or TaKaRa Ex *Taq* enzyme with GC buffer I (TaKaRa, Tokyo). Unless stated otherwise, strain 1147 genomic DNA was used as the PCR template. Construction of the plasmid pXEmetK, containing a *metK*-*xyIE* transcription fusion element, and reverse transcription-PCR (RT-PCR) analysis were performed as described previously (25).

Deletion of *rsmG*. A 1-kb region 5' of *rsmG* was amplified by PCR using primers *rsmG*-F (5'-ACGAGAATTCGCGCAAGTTCATCAACGGTCTG-3') and *rsmG*-R (5'-CTGCGGATCCACGGGGACCGTCTCCGTATG-3'), containing EcoRI and BamHI sites (underlined), respectively. A second 1-kb region 3' of *rsmG* was amplified using primers *rsmG*-F2 (5'-GACGGATCCA TAGCGTCCGGCTTCGCTCGGAT-3') and *rsmG*-R2 (5'-TCGGAAGCTTAC CAGCACGTCGTAGATGGAAG-3'), containing BamHI and HindIII sites (underlined), respectively. The PCR fragments were inserted between the EcoRI and HindIII sites of pK19mob, using three-fragment ligation, and the *rsmG* in-frame deletion construct was shuttled into pGM160::oriT (provided by Haruo Ikeda, Kitasato University) to yield pGMD*rsmG*. *E. coli* ET12567/pUZ8002 was transformed with the recombinant plasmid and mated into *S. coelicolor* 1147. Transformants were selected with thiostrepton and purified by streaking onto thiostrepton-containing plates. To obtain single crossover recombinants, purified transformants were cultured on thiostrepton-containing plates at 37°C (pGM160::oriT carries a temperature-sensitive replicon derived from pSG5 and

cannot replicate at 37°C). Thiostrepton-resistant single-crossover recombinants were subcultured by two rounds of streaking in the absence of thiostrepton at 37°C to obtain double-crossover recombinants in which the delivery plasmid was lost from the cells. Serial dilutions of the resulting spores were plated, and the resulting colonies were tested for thiostrepton sensitivity. Colonies with a thiostrepton-sensitive phenotype were selected, and the correct deletion of *rsmG* was confirmed by PCR using gene-specific primers and DNA sequencing. The Δ *rsmG* mutant strain KO-656 was used for further analysis.

Complementation of the Δ *rsmG* mutation. The *S. coelicolor rsmG* gene was PCR amplified using primers *rsmG*-F and *rsmG*-R2, as described above, and the resulting DNA fragment was cloned between the EcoRI and HindIII sites of the Φ C31-derived integrating plasmid pTYM18 (28) (provided by Hiroyasu Onaka, Toyama Prefectural University) to yield the plasmid pTYM-*rsmG*. *E. coli* ET12567/pUZ8002 cells were transformed with this plasmid and mated into the *S. coelicolor* Δ *rsmG* strain. Transformants were then selected with thiostrepton and used for phenotypic analysis.

Analysis of in vitro methylation profiles of 16S rRNA. 16S rRNAs were extracted from 30S subunits isolated from *S. coelicolor* wild-type and Δ *rsmG* strains. An aliquot (25 μ g) of each extract was digested for 3 h at 37°C with nuclease P1 (3 U) and alkaline phosphatase (0.04 U) in a 25- μ l reaction mixture containing 20 mM HEPES-KOH (pH 7.5). The resulting nucleosides were analyzed by high-performance liquid chromatography (HPLC) using an Inertsil ODS-3 column (250 by 2.1 mm; GL Science, Japan) as described previously (16), except that the pH of solvent A was adjusted to 4.0 instead of 5.3.

Enzyme assays and in vitro protein synthesis. SAM synthetase activity was measured as described previously (25), except that the cells were grown on R5⁻ agar. Cell-free translation of green fluorescent protein (GFP) mRNA and preparation of the S-150 fraction and ribosomes were performed as described previously (13). Western blotting analysis of the RRF was also performed as described previously (13). For sucrose gradient sedimentation analysis, ribosomal 70S complexes were sedimented using a Biocom Piston Gradient Fractionator (Towa Kagaku) equipped with an Atto Bio-Mini UV monitor (27).

RESULTS

Identification of a *str-1* mutation conferring low-level Sm resistance. Because of the inability of conventional genetic strategies to identify mutations conferring low-level Sm resistance, we utilized comparative genome sequencing (CGS), a new method (1) that uses microarray-based DNA sequencing to identify single nucleotide polymorphisms (SNPs) and insertion-deletion sites within the genome. The first phase of CGS (mapping) consists of a genome-wide mutation analysis entailing hybridization of mutant and reference strain genomic DNA to an array containing the M145 reference genome with 7- to 8-bp probe spacing. We utilized the mutant KO-132 (*relA str-1*) strain, showing low-level Sm resistance due to a *str-1* mutation, as the source of mutant genomic DNA, and the M145 strain as the source of reference genomic DNA. Because the *str-1* mutation had previously been mapped to the 7-o'clock position on the *S. coelicolor* chromosome (21), CGS was conducted for 1.2 Mbp around this region. We identified a putative SNP within the gene SCO3885 (Fig. 1), which was confirmed by direct sequencing to be a deletion mutation (deletion of 488A). The homolog of this gene in *E. coli* was described as *gidB* based on its relation to glucose-mediated inhibition of cell division (39), but we recently renamed it rRNA small subunit methyltransferase gene *G* (*rsmG*) (26). Strikingly, six other *str* mutants (Table 1, KO-133 to KO-179) showing low-level Sm resistance were all found to carry a mutation within the *rsmG* gene, strongly suggesting that *rsmG* mutations are responsible for this Sm resistance. These mutations were characterized by the frequent appearance of insertion mutations that resulted in stop codons just downstream of the mutations.

Deletion of the *rsmG* gene. The *str-1* mutation, as well as other *str* mutations (Table 1), was found to activate Act pro-

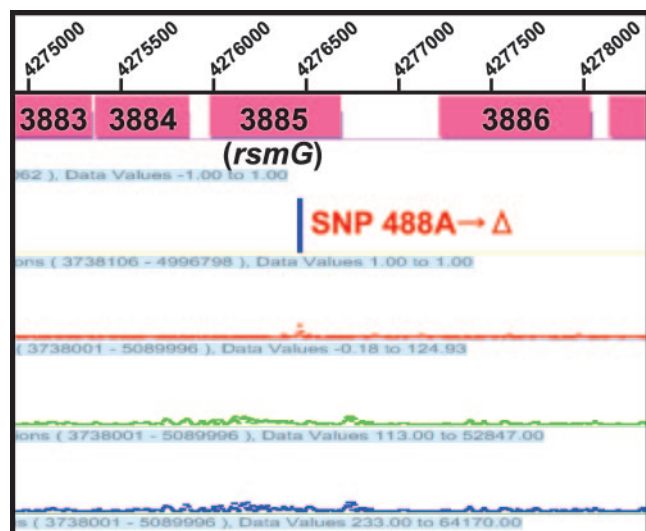


FIG. 1. SignalMap (NimbleGen) representation of CGS analysis of the *str-1* mutant strain KO-132. The lowest two traces show the signal intensities for the M145 wild-type (green) and *str-1* mutant (blue) hybridizations; the red trace above shows their ratio. The top line depicts an SNP confirmed by sequencing.

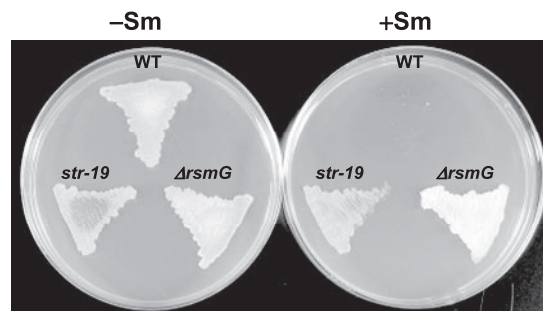


FIG. 2. Effect of deleting the *rsmG* gene on the level of Sm resistance. The 1147 (wild-type [WT]) and KO-656 ($\Delta rsmG$) strains were grown for 2 days on GYM agar with (+SM) or without (-SM) 2 μ g/ml Sm. Strain KO-179 (*rsmG* mutant [*str-19*]) served as a reference.

duction when it was introduced into the wild-type and the *relA* and *brgA* mutant strains, which are otherwise Act deficient (35). To clearly demonstrate that the *rsmG* mutation is responsible for the observed phenotype (i.e., increased resistance to Sm and a greater ability to produce antibiotic), we performed a set of gene disruption experiments. The constructed *rsmG* deletion mutant (strain KO-656 carrying $\Delta rsmG$) showed increased resistance to Sm, as did the *rsmG* (*str-19*) mutant KO-179 strain (Fig. 2), and both showed markedly increased Act production both on plates and in liquid cultures (Fig. 3A

TABLE 1. Location of mutation in the *rsmG* gene and resulting amino acid exchange in RsmG

Strain	Position of mutation in <i>rsmG</i> gene ^a	Amino acid exchange	Resistance to Sm (μ g/ml) ^b	Source
<i>S. coelicolor</i>				
1147 (wild type)	— ^c		1	18
M570 (<i>relA</i>)	—		1	6
KO-132 (<i>relA str-1</i>)	488A \rightarrow Δ	Glu163 \rightarrow Gly (Leu176 \rightarrow stop codon)	5	35
KO-133 (<i>relA str-2</i>)	21C \rightarrow CC	Val10 \rightarrow Cys (Asp16 \rightarrow stop codon)	5	35
KO-135 (<i>relA str-3</i>)	484G \rightarrow GG	Glu163 \rightarrow Gly (frame-shift)	10	35
KO-137 (<i>relA str-4</i>)	21C \rightarrow CC	Val10 \rightarrow Cys (Asp16 \rightarrow stop codon)	5	35
KO-138 (<i>relA str-5</i>)	160C \rightarrow A	His54 \rightarrow Asn	5	35
KO-149 (<i>brgA str-7</i>)	503A \rightarrow G	Gln168 \rightarrow Arg	10	35
KO-179 (<i>str-19</i>)	21C \rightarrow CC	Val10 \rightarrow Cys (Asp16 \rightarrow stop codon)	5	12
KO-178 (<i>str-18</i> ; K88E <i>rpsL</i>)	—		100	12
KO-660	160C \rightarrow T	His54 \rightarrow Tyr	5	This study
KO-661	160C \rightarrow A	His54 \rightarrow Asn	5	This study
KO-662	21C \rightarrow Δ	Val37 \rightarrow stop codon	5	This study
KO-663	627A \rightarrow TA	Val1210 \rightarrow Ser (frame-shift)	5	This study
KO-664	568C \rightarrow T	Gln190 \rightarrow stop codon	5	This study
KO-665	427G \rightarrow A	Ala143 \rightarrow Thr	5	This study
KO-666	21C \rightarrow CC	Val10 \rightarrow Cys (Asp16 \rightarrow stop codon)	5	This study
<i>S. lividans</i>				
1326 (wild type)	—		1.5	18
KO-687	377C \rightarrow Δ	Glu127 \rightarrow Lys (Val139 \rightarrow stop codon)	10	This study
KO-688, 690, 691, 692, 694, 695, 696, 699, 700, 701, 702, 703	21C \rightarrow CC	Val10 \rightarrow Cys (Asp16 \rightarrow stop codon)	10	This study
KO-689	616A \rightarrow AA	Thr206 \rightarrow Asn (frame-shift)	10	This study
KO-693	127G \rightarrow T	Gly43 \rightarrow Cys	10	This study
KO-697	440T \rightarrow C	Leu147 \rightarrow Pro	5	This study
KO-698	368G \rightarrow Δ	Gly134 \rightarrow Ala (Val139 \rightarrow stop codon)	10	This study
KO-704	218A \rightarrow G	Asp73 \rightarrow Gly	10	This study
KO-705	434-439 \rightarrow Δ	145-147Ala, Pro, Leu \rightarrow Val	10	This study

^a Numbering originated from the start codon (GTG) of the open reading frame.

^b Determined 3 days after incubation on GYM agar.

^c Wild-type *rsmG* gene.

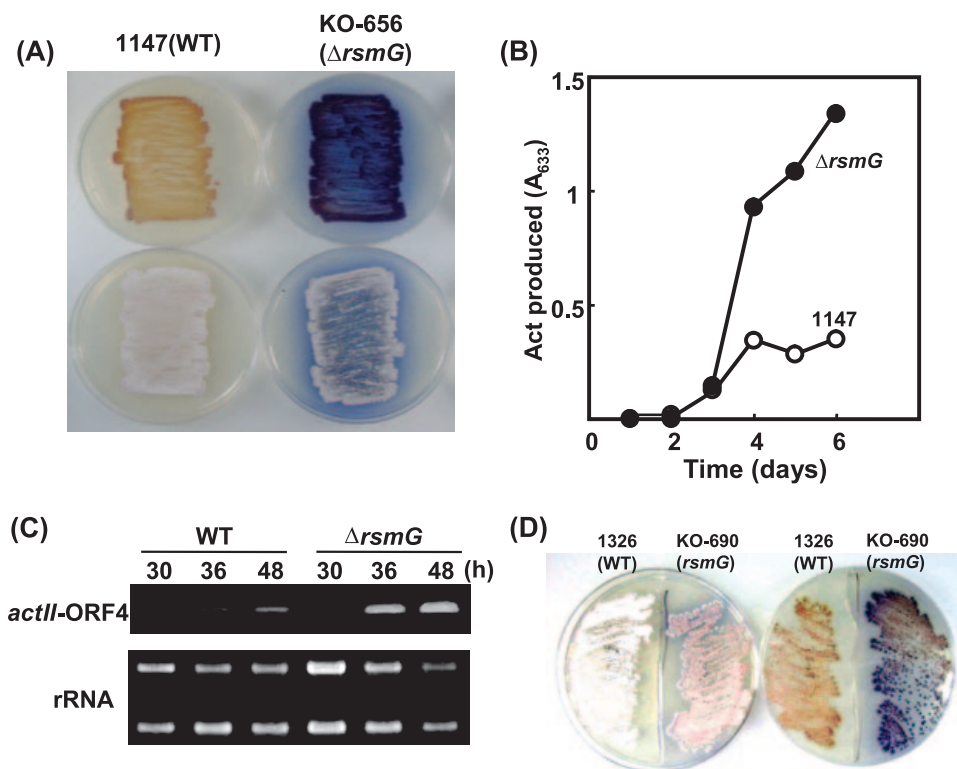


FIG. 3. Effect of deleting the *rsmG* gene on Act production and expression of *actII-ORF4*. (A) Act production by *S. coelicolor* strains 1147 (wild-type [WT]) and KO-656 ($\Delta rsmG$). Upper row shows the reverse side of the plates. Strains were incubated on an R4C agar plate for 4 days. (B) Act production by *S. coelicolor* strains 1147 and KO-656 in R5⁻ liquid medium. (C) Expression of *actII-ORF4* mRNA by cells grown on R4C agar plates for the indicated times, as determined by RT-PCR. (D) Effect of *rsmG* mutation on Act and Red production by *S. lividans* grown on R4C agar plates for 6 days. The upper (left) and reverse (right) sides of the plates are shown. Blue and red represent actinorhodin (Act) and undecylprodigiosin (Red), respectively.

and B), strongly suggesting that these *rsmG* mutations are responsible for the observed phenotypes. Moreover, the introduction of a plasmid containing wild-type *rsmG* into $\Delta rsmG$ cells completely eliminated resistance to Sm and abrogated the positive effect of the $\Delta rsmG$ mutation on antibiotic production (data not shown). The observed positive effect of the $\Delta rsmG$ mutation on Act production was ascribed to transcriptional activation of the pathway-specific positive regulatory gene *actII-ORF4* (Fig. 3C).

Drug-resistant bacteria are generally believed to grow more slowly than susceptible bacteria because mutations that confer resistance also reduce the organism's overall fitness, a phenomenon known as "cost of resistance" (2). However, the *S. coelicolor* $\Delta rsmG$ mutant grew as well as the parent strain in YEME (Fig. 6A), GYM, and R4C media (data not shown).

RsmG functions as a ribosome modulator by targeting 16S rRNA. RsmG protein has a putative SAM binding motif within its primary structure (17) (see Fig. S1 in the supplemental material), and the crystal structure of *E. coli* RsmG shows that this protein contains a SAM-dependent methyltransferase fold within its ternary structure (32). We recently showed that *E. coli* RsmG catalyzes a SAM-dependent 7-methylguanosine (m^7G) modification of G527 in the 16S rRNA (26). By analyzing the nucleosides using reversed-phase HPLC, we confirmed that *S. coelicolor* RsmG also catalyzes an m^7G modification within the *S. coelicolor* 16S rRNA (Fig. 4). As expected,

the peak corresponding to m^7G was absent from the $\Delta rsmG$ mutant, demonstrating that RsmG is responsible for the in vivo methylation of 16S rRNA. Since the m^7G modification is restricted to a single position in *E. coli* 16S rRNA and RsmG has equivalent functions in *E. coli* and *S. coelicolor* (26), these findings suggest that RsmG modifies G518 in *S. coelicolor*, corresponding to G527 in *E. coli*.

The *rsmG* mutant exhibits increased SAM synthetase activity. We previously reported that *S. coelicolor* strain KO-179 (*str-19*), which harbors a Val 10→Cys mutation within *rsmG* (Table 1), exhibits enhanced expression of SAM synthetase, eventually leading to the overproduction of Act (25). This was confirmed by using the $\Delta rsmG$ mutant, in that measurements of enzyme activity revealed, as expected, that the $\Delta rsmG$ mutant displayed a 5- to 10-fold increase in SAM synthetase activity during the late growth phase (Fig. 5A). Furthermore, dramatically enhanced transcription of *metK* (a gene encoding SAM synthetase) accounted for the elevated SAM synthetase activity (Fig. 5B), indicating that by acting at the level of transcription, the *rsmG* mutation has a positive effect on MetK expression.

The $\Delta rsmG$ mutant ribosome exhibits enhanced translational activity. Certain *rpsL* mutations (e.g., the K88E substitution in S12) lead to enhanced protein synthetic activity during the late growth phase, in addition to high-level Sm resistance (13, 27). To test whether *rsmG* mutations also con-

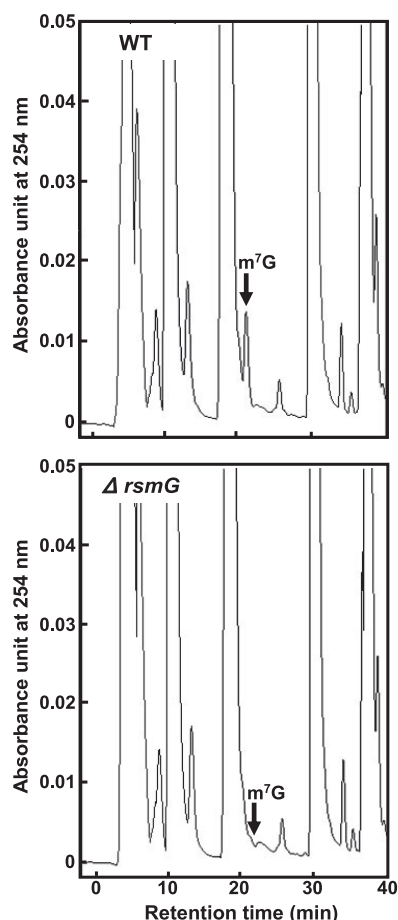


FIG. 4. HPLC profile of 16S rRNA nucleosides from wild-type (upper panel) and $\Delta rsmG$ mutant (lower panel) strains. 16S rRNA was isolated, digested completely with nuclease P1 and alkaline phosphatase, and analyzed by HPLC. The peak position for m^7G was determined using standard m^7G .

fer this phenotype, we measured the *in vitro* translational activity of ribosomes isolated from wild-type and $\Delta rsmG$ cells. Washed ribosomes and the S-150 fraction were prepared from cells grown to early (S_2) stationary phase in YEME medium

(Fig. 6A) and were used to assemble the *in vitro* translation system, using GFP mRNA as a template. In wild-type extracts, the rate of GFP synthesis was maximal during the mid-exponential phase but declined during the stationary phase (13). By contrast, ribosomes isolated from stationary-phase $\Delta rsmG$ cells exhibited a high-level of GFP synthetic activity, as did ribosomes from K88E *rpsL* mutant cells (Fig. 6B); ribosomes prepared from $\Delta rsmG$ cells were 4.5-fold more active than those obtained from wild-type strain 1147 during the stationary phase. Similar results were observed when the activity was compared with cells grown to mid (S_3)-stationary phase (data not shown). Enhanced translational activity during the stationary phase thus appears to be a characteristic property of the $\Delta rsmG$ mutant.

In the K88E *rpsL* mutant, the enhanced protein synthesis activity during the late growth phase was found to reflect the increased stability of the 70S ribosomal complex and the higher levels of RRF in the S-150 fraction (13, 27). Our cross-mixing experiments, however, showed that the S-150 fraction prepared from $\Delta rsmG$ cells did not contribute to the enhancement observed in GFP synthesis (Fig. 6C). Consistent with that result, Western analysis revealed that, unlike that in the K88E *rpsL* mutant, the S-150 fraction in the $\Delta rsmG$ mutant did not show increased RRF levels (Fig. 6D). Moreover, the $\Delta rsmG$ mutation did not lead to increased stability of the 70S ribosomal complex at low (1 mM) Mg^{2+} concentration. By contrast, 70S ribosomal complexes from the K88E *rpsL* mutant showed greater stability than those from wild-type cells or the $\Delta rsmG$ mutant (see Fig. S2 in the supplemental material). These findings indicate that although the *rpsL* and *rsmG* mutant strains both exhibit enhanced protein synthesis activity during late growth phase, the mechanisms underlying this enhanced activity are largely different. Although ribosomes from the $\Delta rsmG$ mutant showed enhanced protein synthesis activity, ribosomes from the wild-type strain, into which a high-copy-number plasmid containing the *metK* gene had been introduced (25), did not (data not shown), indicating that increased SAM synthetase activity did not cause the enhanced protein synthesis activity.

Characterization of the *rsmG* mutation. In bacteria, spontaneous mutations leading to high-level Sm resistance (e.g., a

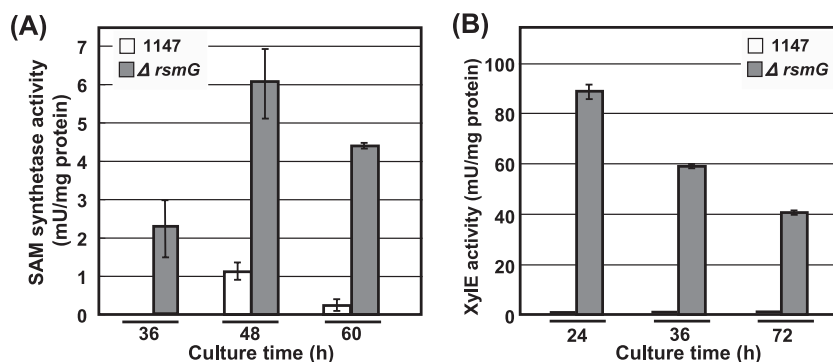


FIG. 5. Effect of deleting *rsmG* on SAM synthetase activity. (A) SAM synthetase activity in wild-type (1147) and $\Delta rsmG$ mutant (KO-656) strains. Cells were grown on $R5^-$ agar covered with cellophane. Samples were taken at the indicated times, and SAM synthetase activity was determined. One unit of activity is defined as the amount of enzyme that changed the optical density at 340 nm at a rate of 12.4/min. (B) Expression of *metK-xylE* fusion element, as determined by quantitative catechol dioxygenase assays in wild-type and $\Delta rsmG$ mutant strains grown on $R5^-$ agar medium. XylE activity was determined as described previously (25).

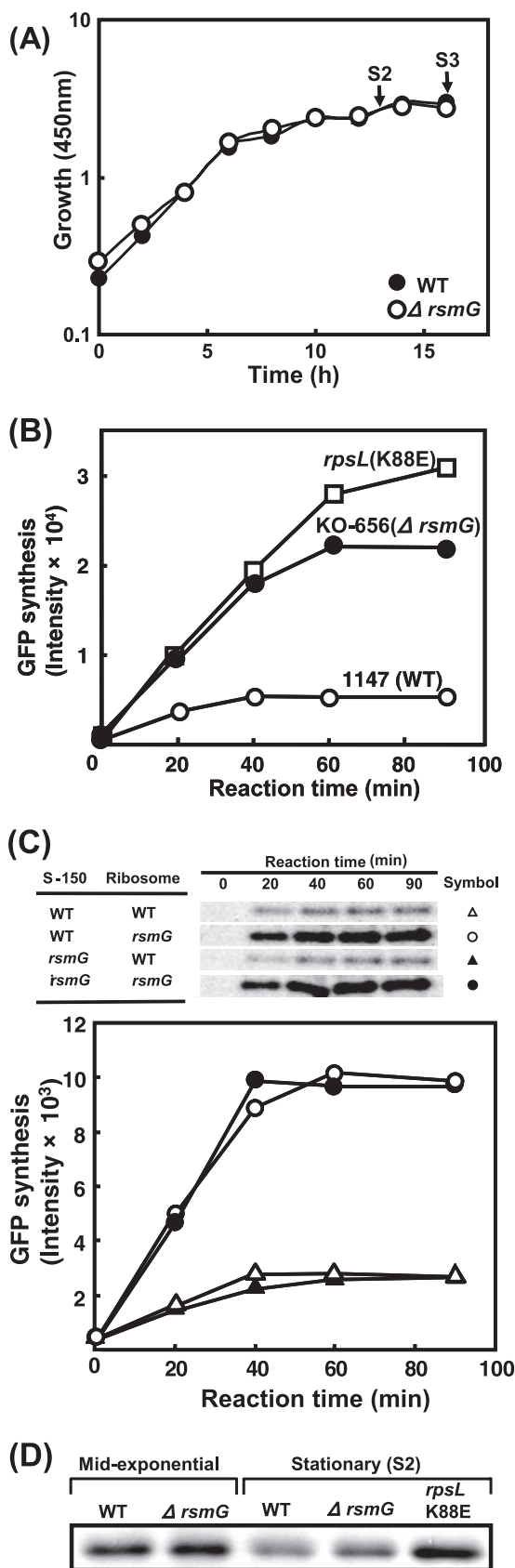


FIG. 6. Profiles of growth and in vitro protein synthesis in wild-type 1147 (WT) and $\Delta rsmG$ (KO-656) strains. (A) Growth in YEME me-

100-fold increase in MIC) generally emerge at a low frequency, with the majority arising within *rpsL* (10). For example, in *S. coelicolor* wild-type strain 1147 (MIC, 1 $\mu\text{g/ml}$), the frequency of emergence of spontaneous mutants with high-level Sm resistance (MIC, $\geq 100 \mu\text{g/ml}$) was as low as 10^{-10} to 3×10^{-10} . By contrast, mutants with low-level Sm resistance (MIC, 5 to 10 $\mu\text{g/ml}$) emerged at much higher frequencies, ranging from 10^{-6} to 8×10^{-6} . Gene sequencing showed that all 18 low-level Sm-resistant mutants harbored a point, deletion, or insertion mutation within *rsmG* and that all produced much more Act on R4C agar medium than did the parental strain 1147 (several isolates [e.g., KO-660 to KO-666] are listed in Table 1). Likewise, *rsmG* mutants of *S. lividans* wild-type strain 1326 (Table 1) produced Act on R4 agar, although the parental organism did not (for example, the *rsmG* mutant strain KO-690 [21C \rightarrow CC, frameshift mutation, C insertion at position 21] in Fig. 3D). Together with the $\Delta rsmG$ mutant, all of these *rsmG* mutants of *S. coelicolor* and *S. lividans* had reduced abilities to form aerial mycelium (and thus, sporulation).

We found that the $\Delta rsmG$ strain produced mutants showing resistance to high-level Sm (100 $\mu\text{g/ml}$) at frequencies ranging from 10^{-8} to 3×10^{-7} , 100- to 1,000-fold higher than the frequencies observed with the wild-type strain 1147. Most (14/20) of these highly resistant $\Delta rsmG$ mutants had *rpsL* mutations, including the previously unreported T41I mutant. Moreover, certain *rsmG rpsL* double mutants (e.g., $\Delta rsmG rpsL$ [K88E] and $\Delta rsmG rpsL$ [K88R]) displayed a greater ability to produce antibiotics than single mutants did (see Table S1 in the supplemental material).

DISCUSSION

The *str-19* and *str-1* mutants have been isolated from *S. coelicolor* wild-type strain 1147 and from the *relA* mutant strain M570, respectively, based on their low-level Sm resistance, accompanied by markedly increased Act production in the former and complete restoration of impaired Act production in the latter (21, 35). Further analysis of these *str* mutants showed that enhanced expression of SAM synthetase caused by the mutation was responsible for the overproduction of Act (25). However, the precise location of the *str* mutations conferring low-level Sm resistance was unknown. In the present

dium at 30°C was monitored by measuring the optical density at 450 nm (OD_{450}). The zero time point represents 22 to 24 h after inoculation of fresh spores, when the OD_{450} was 0.2; "S2" indicates the early stationary phase (see reference 13). (B) In vitro synthesis of GFP using wild-type and $\Delta rsmG$ ribosomes prepared from cells grown to S₂ phase. Strain KO-178 (K88E *rpsL* mutant) served as a reference strain. Equal aliquots (10 μl) of reaction mixture were withdrawn at the indicated times and subjected to electrophoresis in 10% polyacrylamide gels. The intensity of the GFP bands was determined by scanning the fluorographs. (C) Effects on GFP synthesis of cross-mixing the S-150 fractions and ribosomes from wild-type and mutant cells grown in YEME medium to stationary (S₂) phase. Cell-free translation of GFP mRNA was performed as described in the panel B legend. (Upper panel) Fluorographs of synthesized GFP. (Lower panel) Relative levels of GFP synthesis. (D) Expression profile of RRF protein in wild-type (1147) and mutant ($\Delta rsmG$) strains. Strain KO-178 (K88E *rpsL* mutant) was the reference strain.

work, we used the CGS technique to successfully show that this mutation lies within *rsmG* and then characterized the cellular metabolic changes caused by this mutation.

We demonstrated unambiguously that the loss of the m⁷G modification within the 16S rRNA results in resistance to Sm, thereby providing a molecular basis for *rsmG* mutation-induced Sm resistance. The site of the methylation is highly likely to be G518, which corresponds to G527 of *E. coli*, as the RsmG proteins of *S. coelicolor* and *E. coli* appear to be functionally equivalent and, thus, are involved in the same biochemical process (26). It is noteworthy that G527 is located within the 530 loop of 16S rRNA, which appears to play a key role in mediating the accuracy of protein synthesis, and that this invariant nucleotide directly interacts with Sm (5, 8, 24, 30, 31, 37). Consequently, our finding that the failure to methylate G527 residue leads to Sm resistance is striking.

Homologues of *rsmG* are highly conserved among eubacteria, so it was somewhat surprising that despite the apparently important contribution made by RsmG to ribosomal function, the deletion of *rsmG* had no effect on the growth of either *S. coelicolor* (in this study) or *E. coli* (26). The high frequency of emergence of spontaneous *rsmG* mutants is likely due to the dispensability of this gene, allowing cells to remain viable. It is also noteworthy that mutants showing high-level Sm resistance (i.e., *rpsL* mutants) arose at much higher frequencies in *rsmG* mutants than in wild-type cells. We do not know at present the mechanism underlying the high-frequency emergence of high-level Sm resistance, but it is not likely that RsmG functions as an antimutator-like protein, since *rsmG* mutation did not affect the frequency at which mutants resistant to antibiotics other than Sm emerged (26). Nonetheless, our finding that *rsmG rpsL* double mutants have a greater ability to produce antibiotic (see Table S1 in the supplemental material) is intriguing in considering strategies for strain improvement.

We observed a causal relationship between *rsmG* mutation and upregulation of *metK* expression (Fig. 5), which together with earlier work (15, 19, 25) establishes the significance of SAM synthetase activity in initiating antibiotic production in *S. coelicolor* A3(2) and other *Streptomyces* spp. As shown by *metK-xylE* fusion analysis, enhanced expression of MetK protein in the $\Delta rsmG$ disruptant is apparently achieved through the upregulation of transcription (Fig. 5B). It is unclear, however, how the *rsmG* mutation dramatically upregulates *metK* transcription.

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 efficient transcription of its cognate biosynthetic structural genes (3, 11). It is noteworthy that the *rsmG* mutation enhances *actII-ORF4* transcription (Fig. 3C), as it suggests SAM-dependent protein methylation may be involved in controlling the activity of the regulatory proteins encoded by such developmental genes. Alternatively, DNA or RNA methylation may be involved in the expression of these regulatory genes, or SAM itself may be an inducer of Act synthesis. In relation to this notion, recent findings suggest that exogenous addition of SAM enhances the expression of BldK, an oligopeptide transporter important in the regulation of *S. coelicolor* differentiation, as well as the transcription of the

global regulatory *S. griseus* genes *adpA* and *strR*, leading to Sm overproduction (29, 36).

We found that both the $\Delta rsmG$ and K88E *rpsL* mutants exhibited enhanced protein synthetic activity during the late growth phase (Fig. 6), which is consistent with our hypothesis (13) that the capacity of a cell to synthesize protein during late growth phase is indicative of its ability to accelerate the onset of secondary metabolism and to produce biosynthetic enzymes. Although we do not yet know how these mutations mediate preferential gene transcription, it is conceivable that the expression of pathway-specific regulatory genes is governed by higher-order regulatory proteins and that 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. In that regard, it is noteworthy that the 70S ribosome in the $\Delta rsmG$ mutant was not more stable (see Fig. S2 in the supplemental material) and that this mutant did not show increased levels of RRF in the S-150 fraction, all of which would contribute to protein synthetic activity. Apparently, the enhanced protein synthesis observed in these different Sm-resistant mutants (*rpsL* versus *rsmG*) is mediated by largely different mechanisms.

ACKNOWLEDGMENTS

This work was supported by grants to K.O. from the Organized Research Combination System and the Effective Promotion of Joint Research of Special Coordination Funds (the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government).

We are grateful to Hiroyasu Onaka and Haruo Ikeda for their generous gifts of plasmids used here and to the Genefrontier Corp. (Tokyo) for supporting the mutation search using the comparative genome sequencing technique.

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