

# The Gene Encoding RNase III in *Streptomyces coelicolor* Is Transcribed during Exponential Phase and Is Required for Antibiotic Production and for Proper Sporulation<sup>∇</sup>

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**Phenotypic analysis of a constructed RNase III null mutant of *Streptomyces coelicolor* revealed that RNase III is required for both antibiotic production and proper formation of sporulation septa. Transcriptional analysis of the gene encoding RNase III indicated that it is transcribed exclusively during exponential phase as part of a tricistronic message.**

*Streptomyces* is an enormous genus of gram-positive bacteria perhaps best known for the production of secondary metabolites with antibiotic properties. The production of antibiotics is part of the complex *Streptomyces* life cycle and often coincides with differentiation of vegetative hyphae into sporogenous, aerial hyphae (6). When grown in shaken liquid cultures, streptomycetes often begin to produce antibiotics during the transition from exponential to stationary phase (6). *Streptomyces coelicolor* A3(2) has proven to be a useful model organism for studies of antibiotic production because two of the antibiotics that it produces (actinorhodin and undecylprodiginine) are pigmented and thus easily identifiable. Among the many known *S. coelicolor* mutants with defects in the production of antibiotics is the *absB* mutant identified by Champness and coworkers (2, 9, 17). The *S. coelicolor absB* mutant forms aerial hyphae and spores, but it is unable to produce actinorhodin, the prodiginines, and the calcium-dependent antibiotic. This mutant was found to harbor a loss-of-function mutation in a gene encoding an ortholog of *Escherichia coli* RNase III (*rnc*), a double-stranded RNA-specific endoribonuclease (5). In *E. coli*, this enzyme is known to process rRNA, bacteriophage RNA, and a few mRNAs, including the dicistronic transcript encoding *rpsO* and *pnp* (4, 18, 21). By analogy with *E. coli*, *S. coelicolor* RNase III is presumed to process rRNA and has been shown to cleave the *rpsO-pnp* transcript in vitro (10). A clear explanation for the requirement for RNase III for antibiotic production in *S. coelicolor* has not yet been established (1, 10).

Here, we report the construction of an *S. coelicolor rnc* null mutant and phenotypic characterization of this mutant. In addition, we used high-resolution S1 nuclease mapping experiments to determine when and how the gene encoding RNase III gene is transcribed.

**Phenotypic analysis of an *S. coelicolor rnc* null mutant.** PCR-targeted mutagenesis was used for replacement of the *S. coelicolor rnc* gene with an apramycin resistance marker, *apr* (13). The requisite PCR product was amplified from the

apramycin resistance gene insert of pIJ773 using primers SCO5572 KO FOR (AGGTCCTCGAGGTCTGAGCGGC TGGTGAGAGGCACTGTGATTCCGGGGATCCGTCG ACC) and SCO5572 REV (TGCCGGGGCGGGCGTTCG GACCGTGCGGTGGACGGGTCATGTAGGCTGGAGC TGCTTC). The PCR product was introduced into *E. coli* BW25113/pIJ790 harboring cosmid St7A1 and expressing  $\lambda$  RED recombinase. The resultant recombinant cosmid, St7A1  $\Delta rnc::apr$ , was introduced via *E. coli* strain ET12567/pUZ8002 into *S. coelicolor* M600 by conjugation. M600 is a plasmid-free derivative of the wild-type strain (15). Exconjugants lacking the *rnc* gene were identified by selection for apramycin resistance and kanamycin sensitivity. Gene replacement was confirmed by PCR analysis of both the recombinant cosmid and genomic DNA from the null mutant, *S. coelicolor* J3410  $\Delta rnc::apr$ .

As expected, the phenotype of the constructed *S. coelicolor rnc* null mutant closely resembled that reported for the *absB* mutant. The null mutant did not produce actinorhodin or the prodiginines when it was grown on any of the nine different solid media tested (Fig. 1). These results are notable because the phenotypes of other mutants with pleiotropic defects in antibiotic production are dependent on the growth medium (8). Furthermore, the null mutant did not produce antibiotics when it was grown in SMM, a minimal liquid medium optimized for antibiotic production in *S. coelicolor* (15). After 48 h of growth in this medium, the null mutant did not contain measurable quantities of actinorhodin or the prodiginines, while wild-type *S. coelicolor* grown under identical conditions produced these antibiotics copiously (data not shown). In this medium, the null mutant and wild-type strain have comparable growth kinetics, but the null mutant accumulates about 20% more biomass than the parent strain by late stationary phase (Fig. 2).

In addition to the defect in antibiotic production, the constructed *rnc* null mutant and the *absB* mutant are distinguished by colonies that are about one-half the diameter of wild-type *S. coelicolor* colonies (2). The surfaces of the null mutant colonies were analyzed by scanning and transmission electron microscopy and by fluorescence microscopy. In electron micrographs of the null mutant colonies, it was readily apparent that there was a defect in sporulation (Fig. 3). Whereas the wild-type

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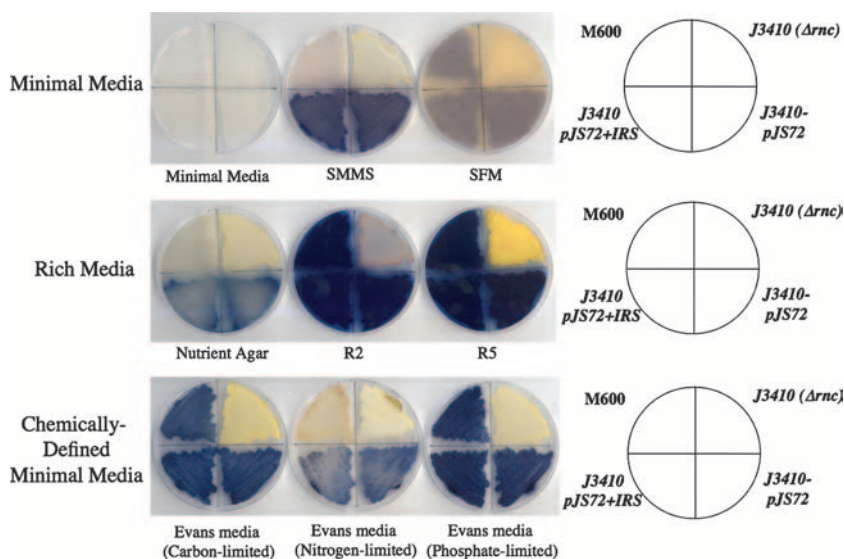


FIG. 1. The *S. coelicolor mc* gene is absolutely required for antibiotic production. Wild-type *S. coelicolor* M600, *S. coelicolor* J3410  $\Delta rnc::apr$ , *S. coelicolor* J3410/pJS72, and *S. coelicolor* J3410/pJS72+IRS (clockwise from the top left) were grown on minimal, complex, and chemically defined Evans media (15, 20). Ectopic expression of the *S. coelicolor mc* gene with and without the 3' inverted repeat sequence (IRS) causes overproduction of actinorhodin on SMMS, R2, and all chemically defined Evans media.

strain produced spores of uniform size and shape, the *mc* null mutant produced both elongated and abnormally small spores. The irregularity in size indicates that RNase III is required for proper formation of sporulation septa in the aerial hyphae. It is noteworthy that both the small and elongated spores of the *mc* null mutant contained DNA, as observed in fluorescent micrographs of aerial hyphae stained with propidium iodide or 4',6-diamidino-2-phenylindole (DAPI) (data not shown). Based on spore counts followed by quantification of CFU on solid media, more than 90% of the null mutant spores were indeed viable.

The *S. coelicolor mc* gene (SCO5572) and the two upstream genes (SCO5570 and SCO5571) lie in the same orientation and are separated by only 2 and 19 bp, respectively, suggesting that

the three genes might form an operon (Fig. 4A). SCO5570 encodes a hypothetical protein with a predicted metal and nucleic acid binding domain, and SCO5571 encodes ribosomal protein L32 (*rpmF*) (5; <http://streptomyces.org.uk>). For complementation, restriction fragments containing only *mc* (SCO5572) or *mc* together with the two upstream genes (SCO5570 and SCO5571) (Fig. 4A) were cloned into the EcoRV site of pMS81, a hygromycin-resistant vector that integrates site specifically into the chromosome at the  $\phi$ BT1 *attP* site (12). These constructs were introduced into the *S. coelicolor mc* null mutant via *E. coli* strain ET12567/pUZ8002 by conjugation. Only provision of the *mc* gene in *cis* with the two upstream genes restored antibiotic production (Fig. 4B), sug-

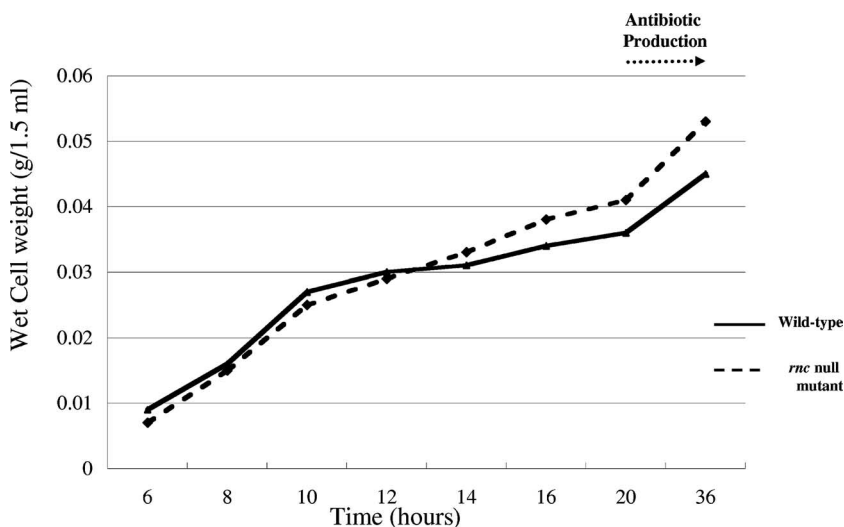
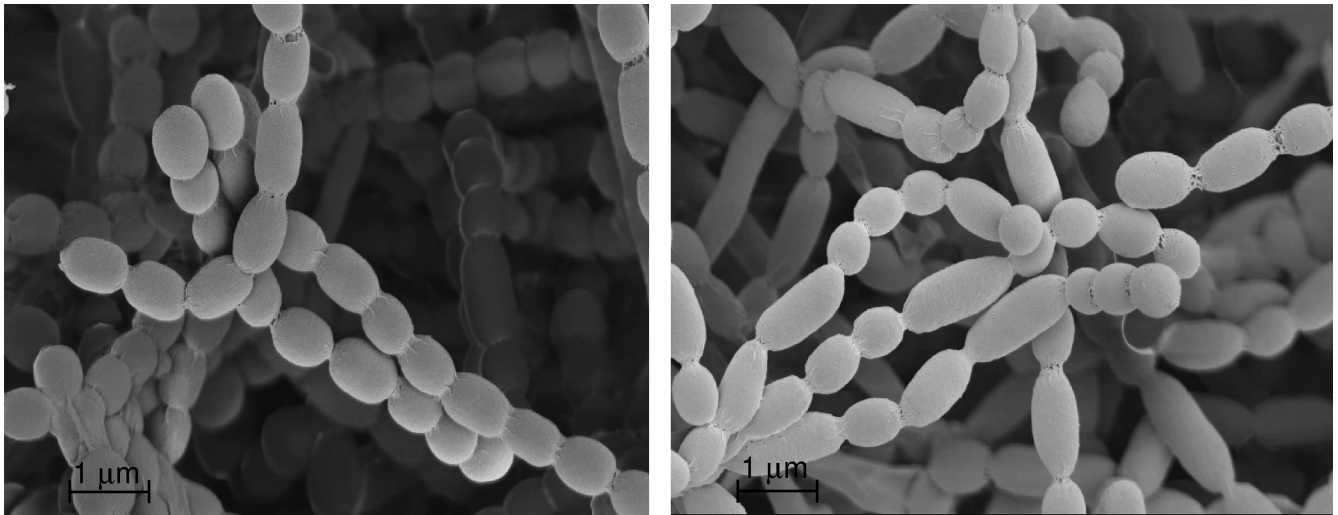


FIG. 2. Comparison of growth kinetics of wild-type *S. coelicolor* and the *mc* null mutant. Both strains were grown as shaken liquid cultures in SMM from an initial inoculum of  $\sim 10^8$  CFU/ml for 36 h at 30°C. Production of the pigmented antibiotics was visible by 24 h in the wild-type cultures. The growth curves reflect the averages of three separate experiments.



*S. coelicolor* M600

*S. coelicolor* J3410 ( $\Delta rnc$ )

FIG. 3. The *S. coelicolor mc* gene is required for proper formation of sporulation septa: scanning electron micrographs of the *mc* null mutant and the wild-type strain grown for 5 days on MS medium at 30°C.

gesting that the *mc* gene is the last gene in a tricistronic message. Additional complementation experiments were designed to verify that the *mc* gene alone was sufficient to restore the wild-type phenotype. The *S. coelicolor mc* gene (both with and without its 3' inverted repeat sequence) was placed under the control of the strong, constitutive *ermE* promoter in the hygromycin-resistant, *attP* integrative vector pIJ10275 (H.-J. Hong, unpublished). The gene was amplified by PCR from cosmid St7A1 using primer SCO5572 FOR (AACATATGTCAGT

CCCCAAGAAGGC) and primer SCO5572 REV (AAGGATC CAAGCTTTCAGGCGGAGGCGGA) or SCO5572IRS REV (AAGGATCCAAGCTTGCCGGTGGATGAGCGA) (engineered NdeI and BamHI sites are underlined), cloned into the EcoRV site of pBluescript KS+ (Stratagene), and sequenced. NdeI and XhoI fragments from the cloning constructs were ligated into complementary sites of pIJ10275. The resulting plasmids (pJS72 and pJS72IRS) were introduced into the *mc* null mutant of *S. coelicolor* by conjugation. In the null mutant,

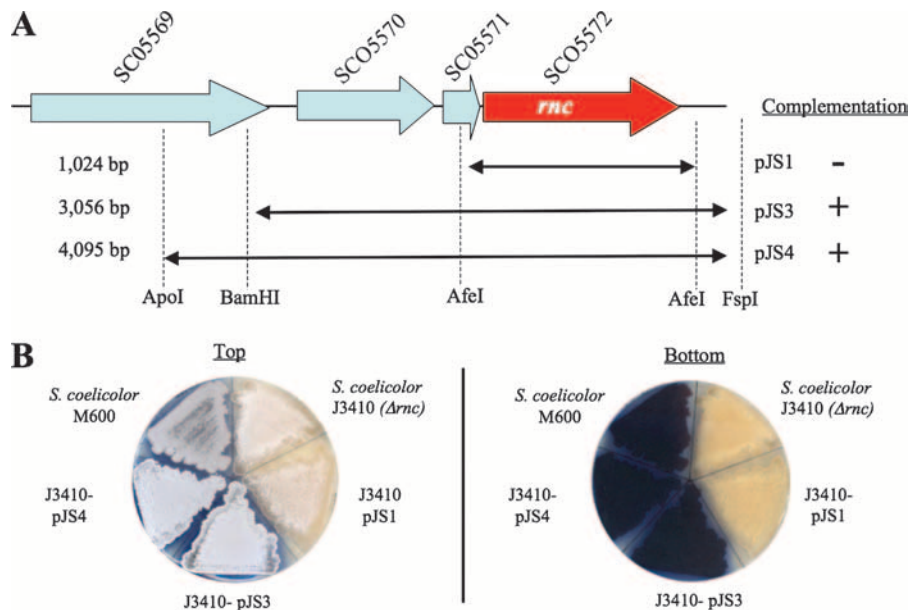


FIG. 4. Complementation of the *S. coelicolor mc* null mutant. (A) Inserts of *S. coelicolor* J3410 ( $\Delta rnc$ ) complementing clones and comparison to the equivalent regions of the *S. coelicolor* chromosome (5; <http://streptomyces.org.uk>). *mc* is SCO5572. SCO5570 encodes a protein with a predicted metal and nucleic acid binding domain, and SCO5571 encodes ribosomal protein L32 (*rpmF*). The vector in all of the clones is pMS81 (12). (B) Top and bottom views of plates showing complementation of *S. coelicolor* J3410 ( $\Delta rnc$ ) by pJS3 and pJS4 but not by pJS1. The cultures were grown on Difco nutrient agar for 4 days at 30°C.

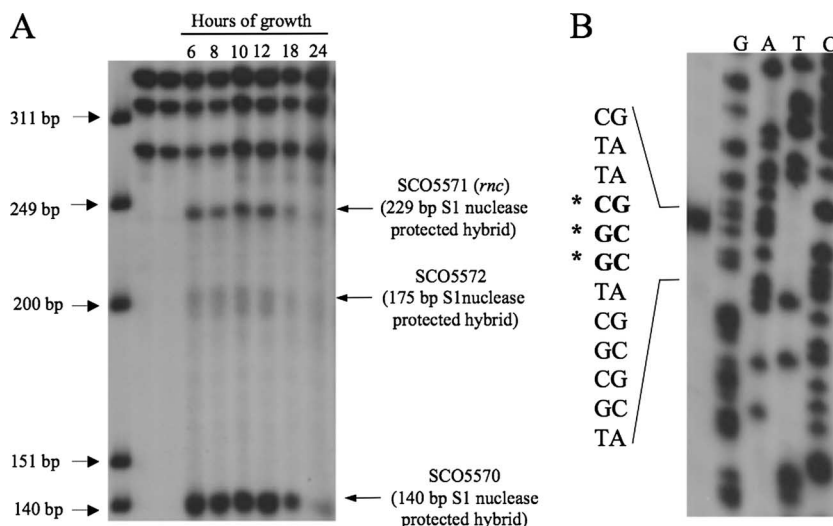


FIG. 5. Transcriptional analysis of the *S. coelicolor mc* (SCO5572) gene and the two upstream genes, SCO5570 and SCO5571. (A) S1 nuclease mapping of the SCO5570, SCO5571, and SCO5572 mRNA transcripts among total RNA isolated at various time points from shaken liquid cultures of *S. coelicolor* M600 grown in liquid SMM at 30°C. The lanes are numbered from left to right. Lane 1 contained the  $\phi$ X174/HinfI ladder. Lane 2 contained the undigested probes including 85-bp nonhomologous ends derived from the T3 site of pBluescript SCO5570 (428 bp), SCO5571 (320 bp), and SCO5572 (274 bp). Lane 3 contained the digested probes mixed with a tRNA control. Lanes 4 to 9 contained the S1 nuclease-protected probe-RNA hybrids. The three probes were simultaneously hybridized to total RNA. (B) S1 nuclease mapping of the transcriptional start site of SCO5570 was conducted using a 450-bp PCR product uniquely labeled at the 5' end. The asterisks indicate the probable transcriptional start sites. Lanes G, A, T, and C contained sequence ladders derived from the same labeled primer that was used to generate the PCR product.

constitutive expression of the *mc* gene not only restored antibiotic production but also caused overproduction of actinorhodin on certain solid media (Fig. 1). All complementation plasmids that restored normal antibiotic production to the *mc* null mutant also restored proper formation of sporulation septa (data not shown).

**Transcriptional analysis of the *S. coelicolor* gene encoding RNase III.** The results of the complementation experiments suggested that the *mc* gene forms part of an operon (Fig. 4). To confirm this and to assess the timing of transcription, high-resolution S1 nuclease protection analysis of RNA isolated from *S. coelicolor* grown in liquid SMM (Fig. 2) and on R2YE solid medium (data not shown) was performed. RNA was isolated at time points in the exponential, transition, and stationary phases of growth by using previously described methods (19). In each experiment, 30  $\mu$ g of RNA was hybridized with 1 fmol ( $10^3$  Cerenkov counts  $\text{min}^{-1}$ ) of the probe (15, 19). The probes were amplified from the cosmid St7A1 template using the following primer pairs: SCO5570 S1-FOR (GACCA GCCTCTTCGACACCAG) and SCO5570 S1-REV (GCTCGT GCGTGTCGACAC), SCO5571 S1-FOR (GCTTGTTGTAA GTGCCGCAAGC) and SCO5571 S1-REV (GCTTGTTGTA AGTGCCGCAAGC), and SCO5572 S1-FOR (TTACAACA AGCGCCAGGTCC) and SCO5572 S1-REV (GTAGGAAC GGTGGGTCAGTG). The PCR products were cloned into pBluescript KS+ (Stratagene) to enable preparation of S1 nuclease probes with 75-bp 3' nonhomologous ends as internal controls. Thus, the probes were amplified by PCR using the cognate reverse primers  $^{32}\text{P}$  labeled at the 5' end with T4 kinase and  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  and the pBluescript T3-specific primer (GCGCAATTAACCCTCACTAAAGGG). When annealed to mRNA, the probes spanning the region surrounding the translation start sites of the *mc* gene and the upstream gene

SCO5571 encoding ribosomal protein L32 were fully protected from the S1 nuclease; however, significant S1 nuclease digestion of the SCO5570 probe was observed, yielding a 140-nucleotide protected DNA fragment (Fig. 5A). These results indicate that the three genes are cotranscribed in the exponential phase and that the transcription start site is upstream of SCO5570. Electrophoresis of the 140-nucleotide protected DNA fragment with a sequencing ladder (generated using the same radiolabeled oligonucleotide primer that was used to generate the S1 nuclease mapping probe) indicated that the most likely transcriptional start site is 82 bp upstream of the SCO5570 start codon (Fig. 5B). The putative  $-35$  and  $-10$  sequences of the SCO5570 promoter are TTGGGC-N<sub>18</sub>-TATCCT. This promoter is very likely to be recognized by the HrdB principal essential sigma factor (7).

**Concluding remarks.** Although it does not catalyze biosynthetic reactions, RNase III is an essential enzyme for antibiotic production in *S. coelicolor*. The results reported here indicate that this enzyme is also required for proper formation of sporulation septa. These observations are consistent with pleiotropic effects; indeed, the *E. coli mc* gene influences the level of  $\sim 10\%$  of all cellular proteins (11). The apparent exponential-phase transcription of the *S. coelicolor mc* gene and cotranscription with the upstream gene encoding ribosomal protein L32 are consistent with a role for *S. coelicolor* RNase III in the processing of rRNA, as is the case for its ortholog in *E. coli*. Curiously, the nearly identical vegetative growth kinetics of the *S. coelicolor mc* null mutant and the wild-type strain in liquid culture raise questions about the significance of rRNA processing by RNase III in *S. coelicolor*. It is possible that RNase III is one of many cooperative proteins that regulate antibiotic production (6, 14, 16). For instance, RNase III could catalyze a hydrolysis reaction that inactivates an mRNA en-

coding a repressor or activates a transcript encoding an activator of antibiotic production (1, 10). Alternatively, the phenotype of the *S. coelicolor rnc* null mutant could be explained by incomplete processing of rRNA that yields ribosomes lacking the requisite processivity for translation of the atypically long mRNAs of secondary metabolism genes in *S. coelicolor* (5; <http://streptomyces.org.uk>). Biochemical experiments are needed to distinguish between these two possibilities.

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