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Coenzyme B12 Controls Transcription of the *Streptomyces* Class Ia Ribonucleotide Reductase *nrdABS* Operon via a Riboswitch Mechanism†

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Ribonucleotide reductases (RNRs) catalyze the conversion of ribonucleotides to deoxyribonucleotides and are essential for de novo DNA synthesis and repair. Streptomycetes contain genes coding for two RNRs. The class Ia RNR is oxygen dependent, and the class II RNR is oxygen independent and requires coenzyme B12. Either RNR is sufficient for vegetative growth. We show here that the *Streptomyces coelicolor* M145 *nrdABS* genes encoding the class Ia RNR are regulated by coenzyme B12. The 5′-untranslated region of *nrdABS* contains a 123-nucleotide B12 riboswitch. Similar B12 riboswitches are present in the corresponding regions of eight other *S. coelicolor* genes. The effect of B12 on growth and *nrdABS* transcription was examined in a mutant in which the *nrdJ* gene, encoding the class II RNR, was deleted. B12 concentrations of just 1 μg/liter completely inhibited growth of the NrdJ mutant strain. Likewise, B12 significantly reduced *nrdABS* transcription. To further explore the mechanism of B12 repression, we isolated in the *nrdJ* deletion strain mutants that are insensitive to B12 inhibition of growth. Two classes of mutations were found to map to the B12 riboswitch. Both conferred resistance to B12 inhibition of *nrdABS* transcription and are likely to affect B12 binding. These results establish that B12 regulates overall RNR expression in reciprocal ways, by riboswitch regulation of the class Ia RNR *nrdABS* genes and by serving as a cofactor for the class II RNR.

Ribonucleotide reductases (RNRs) provide the building blocks for DNA synthesis and repair in all living cells (22). They are essential because they are the only known de novo pathway for the biosynthesis of deoxyribonucleotides, the immediate precursors of DNA synthesis. Three major classes of RNRs are known. Class I RNRs are oxygen dependent and are divided into subclasses Ia and Ib. Class Ia NrdAB enzymes occur in eukaryotes and bacteria and some viruses and consist of two subunits, with each subunit itself a dimer. The larger catalytic subunit R1 (α_2), encoded by *nrdA*, contains the active site and allosteric effector binding sites. The smaller subunit R2 (β_2), encoded by *nrdB*, contains a dinuclear iron center that generates, in the presence of oxygen, a stable tyrosyl radical required for enzyme activity. Class Ib NrdEF RNRs are confined to bacteria and are distinguished from the Ia enzymes in certain features of their allosteric regulation. Class II NrdJ RNRs are oxygen-independent enzymes that occur in aerobic and anaerobic bacteria. They mainly consist of a single polypeptide, encoded by *nrdJ*, that generates a transient 5′-deoxyadenosyl radical through homolytic cleavage of adenosylcobalamin (coenzyme B12). Class III NrdDG RNRs are present in anaerobic bacteria and use *S*-adenosylmethionine and an iron sulfur cluster to create a stable glycyl radical. All three RNRs subsequently create a protein cysteinyl radical that initiates reduction of ribonucleotides, and all employ allosteric mechanisms to ensure the balanced formation of all four deoxyribonucleotides (5, 9).

While eukaryotes employ just the class Ia RNR, many bacteria and archaea possess more than one kind of RNR. This presumably reflects their evolutionary history and varied life cycles (9, 10, 19, 28, 29). In some cases the rationale for having more than one RNR system is evident. Facultative aerobes possess RNRs that are individually dedicated to aerobic and anaerobic growth (13). Other bacteria, such as the actinomycetes, frequently contain two RNRs, one oxygen dependent and the other oxygen independent, either of which can function in aerobic conditions (3, 10, 29). In this case the particular role of each RNR is not obvious. We have chosen to address this issue in streptomycetes, gram-positive high-G+C aerobic bacteria that belong to the actinomycetes. *Streptomyces* spp. have been intensively studied for the remarkable variety of valuable metabolites they produce and for their complex life cycle (4, 8). *Streptomyces* spp. contain class Ia and class II RNRs (3). In *Streptomyces coelicolor* the class Ia RNR *nrdAB* genes are cotranscribed with *nrdS*, which encodes an AraC-like regulatory protein; likewise, the *S. coelicolor* class II RNR *nrdJ* gene forms an operon with a regulatory gene *nrdR*. Either RNR is sufficient for normal vegetative growth (2). Previously we showed that NrdR, the protein product of *nrdR*, regulates transcription of both sets of genes by showing that the level of *nrdABS* and *nrdRJ* mRNAs was significantly increased when *nrdR* was deleted. Likewise, coenzyme B12, an essential cofactor for the class II RNR, is known to regulate transcription of both sets of genes since mRNA levels were markedly increased when B12 biosynthesis was abolished by a mutation in the *cobN* gene (2).

In this work we examine the role of coenzyme B12 on transcriptional regulation of the *S. coelicolor nrdABS* operon. These studies stem from the fact that we previously noted the presence of a consensus B12 genetic control element in the approximately 350-nucleotide (nt) 5′-untranslated leader region (UTR) of the *S. coelicolor nrdABS* mRNA and speculated that its function is to enable B12 to control *nrdABS* expression

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(2). The B12 element is one of a growing number of genetic control elements, termed riboswitches, that modulate gene expression in bacteria through binding of small molecules (such as vitamins, amino acids, and purines) to the 5′-UTR of mRNA to generate alternative secondary structures (1, 14, 18, 31). The RNA sensor element embedded in the leader sequences binds the metabolite, causing repression or activation of their cognate genes (18, 31, 32). In *Escherichia coli* and *Salmonella enterica* serovar Typhimurium, B12 represses translation of genes coding for B12 biosynthesis (*cob*) and transport (*btuB*) (12, 23). A conserved motif in the 5′-UTRs called a B12 box (6) prevents ribosome access to the mRNA to inhibit translation (16, 17, 20, 21). In *Bacillus subtilis* B12 is reported to terminate transcription of *yvrC*, part of a four-gene operon that appears to encode proteins involved in metal import and processing (14). Biochemical and genetic analysis has confirmed that B12 binds directly to the *btuB* 5′-UTR RNA (14, 16). Comparative genome analysis has identified additional B12 riboswitches in a variety of other genes in diverse bacteria (reviewed in references 15 and 31). These include genes encoding the B12-independent class Ia NrdAB, class Ib NrdIEF, and class III NrdDG ribonucleotide reductases and the B12-independent methionine synthetase (MetE). It is noteworthy that *Streptomyces* and some other bacteria possess both B12-independent NrdAB and MetE enzymes and alternative B12-dependent NrdJ and MetH enzymes. Recently it was proposed that in bacteria which possess both B12-dependent and B12-independent isozymes, the B12-independent enzymes are regulated by B12 riboswitches (30). We have previously shown in *Streptomyces* (which synthesizes B12) that the class II B12-dependent RNR is transcribed at a much higher level than the class Ia RNR and is the primary source of RNR activity in vegetatively growing cells (2, 3). In this work we provide experimental evidence that the *S. coelicolor* class Ia B12-independent RNR is controlled by a genetic riboswitch that functions, in the presence of B12, to inhibit expression of the class Ia RNR genes. We also describe the isolation and characterization of mutations in the *nrdABS* 5′-UTR that relieve B12 inhibition.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Streptomyces coelicolor* strain M145 is referred to as the wild type (11). Derivative strains containing mutations in *nrd* genes have been previously described (2). M145Δ*nrdJ*::*apr773* contains an apramycin resistance cassette in place of *nrdJ* and is denoted by M145Δ*nrdJ*; M145Δ*nrdB*::*apr773* contains an apramycin resistance cassette in place of *nrdB* and is denoted by M145Δ*nrdB*; KF61 is M145 containing a Tn*4561* transposon insertion in the *cobN* gene (provided by Tobias Kieser), and M145Δ*nrdJcobN* was obtained in this study by protoplast fusion (11).

Media for growth of *S. coelicolor* M145 were as follows. MS agar (11) was used to prepare spore suspensions, MY9 agar is MY agar (26) supplemented with Middlebrook 7H9 Broth (4.7 g/l) and was used for growth in solid medium, and YEME medium (11) was used for growth in liquid. Cultures of *S. coelicolor* M145 were grown essentially as described (27). When needed, media were supplemented with apramycin (50 μg/ml; Sigma) or viomycin (30 μg/ml; gift of Tobias Kieser). Adenosylcobalamin was from Sigma.

Northern blot analysis. Pregerminated spores of *S. coelicolor* strains were grown exponentially in YEME medium to an optical density at 450 nm (OD_{450}) of ~0.4 and divided into two equal parts. B12 was added to one half to a final concentration of 50 μg/liter, and incubation continued for 30 min. Cells (25 ml) were collected by centrifugation and washed with TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA). About 300 mg (wet wt) cells were obtained from 25 ml culture and stored at −20°C. Total RNA was isolated from about 200 mg (wet wt) of cells according to the modified Kirby procedure (11). The TPNS reagent was replaced with *N*-lauryl sarcosine (Sigma L-9150). RNA concentrations were determined by A_{260} measurements, and RNA integrity was analyzed by agarose/formaldehyde gel electrophoresis (25). Northern blot analysis was performed as described previously (2). RNA (5 μg) was electrophoresed in an agarose/formaldehyde gel and transferred to NytranN nylon membranes (Schleicher & Schuell). Internal *nrdB* and *hrdB* DNA fragments were amplified by PCR and labeled with the PCR DIG Probe Synthesis kit (Roche). PCR primers were as follows: forward nrdB1 (5′-TTCCGGGACGAGACGATGCACATG) and reverse nrdB2 (5′-GGGCGCCGCTCGAAGAAGTT) producing a fragment of 331 bp; forward hrdB1 (5′-CTCTGTCATGGCGCTCATTG) and reverse hrdB2 (5′-AGGTAGTCCTTGACCGGGTC) producing a fragment of 605 bp.

Quantitative RT-PCR. Total RNA for reverse transcription (RT)-PCR analysis was isolated from exponentially growing cultures using the RNA-Spin total RNA extraction kit (Intron Biotechnology). Frozen cells (20 mg) were suspended in 200 μl TE buffer and treated for 15 min with 10 mg/ml lysozyme (Sigma) with intermittent vigorous mixing and shearing in an 18-gauge needle. Cell lysates were loaded on columns, and RNA was purified according to the manufacturer's instructions. RNA yields were 25 to 30 μg per column. Removal of trace amounts of DNA was carried out by using 10 U of RQ1 RNase-free DNase (Promega) in a 100-μl reaction mixture containing 50 μg RNA and incubating the mixture for 30 min at room temperature. The reaction was stopped by phenol extraction, and then RNA was collected by ethanol precipitation.

RT reactions and PCR amplification of cDNA were performed as previously described (3). cDNA was made in a 20-μl reaction mixture containing 2 μg RNA, 10 U avian myeloblastosis virus reverse transcriptase (Promega) and 20 pmol of reverse primer, nrdB2, or hrdB2. Amplification of *nrdB* and *hrdB* cDNAs was carried out with the forward and reverse primers described above. For *nrdB*, the RT reaction and the annealing step in the PCR were carried out at 54°C, and for *hrdB* the RT reaction was carried out at 56°C and the annealing step in the PCR was performed at 62°C.

Quantitative real time RT-PCR was carried out with the LightCycler system (Roche Applied Science) using LightCycler FastStart Master SYBR green I as previously described (2).

Reaction mixtures (20 μl) contained 0.5 μM forward and reverse primers, 3.5 mM $MgCl_2$, 5% dimethyl sulfoxide, and 2 μl of 1:3 dilutions of cDNA as template. Known amounts of specific cDNAs were used as standards for quantitative reference. Forty cycles of amplification were performed. For *nrdB* and *hrdB*, cDNA amplification conditions were as follows: annealing for 10 s at 55°C and 62°C, respectively, and extension for 16 s and 26 s, respectively, at 72°C. Melting curve analysis was performed in the range of 70 to 98°C. The melting temperatures of the *nrdB* and *hrdB* DNA fragments were 92°C and 94°C, respectively; the melting temperatures of the primer-dimer complexes were 80°C and 76°C, respectively. Fluorescence was determined at 86°C in each case.

Sequence analysis and database searches. Sequence entry, primary analysis, and open reading frame searches were performed using the NCBI server ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and the Clone Manager 7 program (Scientific & Educational Software, Durham, NC). BLAST searches of *Streptomyces avermitilis* MA-4680, *Streptomyces coelicolor* A3(2), and *Streptomyces scabies* 87.22 genomes were prepared at http://avermitilis.ls.kitasato-u.ac.jp/, http://www.sanger.ac.uk/Projects/S_coelicolor/, and http://www.sanger.ac.uk/Projects/S_scabies/, respectively. Systematic analysis of the cobalamin riboswitch motif (accession number RF00174) and other noncoding RNA families in *Streptomyces* and other bacterial genomes were performed by use of http://www.sanger.ac.uk/Software/Rfam/search.shtml (7).

Nucleotide sequencing. Nucleotide sequences were determined using an ABI Prism 3100 genetic analyzer (Applied Biosystems) and the Big Dye Terminator cycle sequencing kit (Applied Biosystems), as recommended by the manufacturer, except that 5% (vol/vol) dimethyl sulfoxide was added to each reaction mixture. Sequences were determined for both strands.

RESULTS

The 5′-untranslated region of the *nrdABS* mRNA contains a B12 riboswitch. Streptomycetes possess two functional RNR systems that are differentially expressed during vegetative growth (3). Northern analysis showed that transcription of the class II RNR *nrdRJ* genes of *S. coelicolor* was readily detected in vegetative growth, whereas transcription of the class Ia RNR *nrdABS* genes was barely detectable. S1 and RT-PCR analysis of total RNA revealed a long (~350-nt) 5′-UTR upstream of the *nrdA*

A

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SCO5228 --350-- nrdA (SCO5226) -- nrdB (SCO5225) --271-- nrdS (SCO5224)
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B

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                                                    -35                         -10
SCO nrdA   CCGCGCCGGGACACAACATCTGGGGGTGCTCGCGTCCCCCGGCACAAGATGTATGCTCAT
SAV nrdA   CCG--ACGGGACACAAGATGTGGGGGTGCCACCGGGGCCCGGCACAAGATGTATGCTCAT
SSC nrdA   CCG-AACGCGACACAACATGTGGGGGTGACACCGCGCCCCGGCACTAGATGTATGCTCAT
SLI nrdA   nnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnCCGGCACAAGATGTATGCTCGT

           tsp P1              P3               P3'   P4   P4'
SCO nrdA   GCTCGCTGTCGCCGCAGGGGAATCCGGTGCGAATCCGGAACTGTCCCGCAACGGTGTACT
SAV nrdA   GCTCGCTGTCGCCGCAGGGGAATCCGGTGCGAATCCGGAACTGTCCCGCAACGGTGTACT
SSC nrdA   GCTCGCTGTCGCCGCAGGGGAATCCGGTGCGAATCCGGAACTGTCCCGCAACGGTGTACT
SLI nrdA   CCTCGCTGTCGTCGCAGGGGAATCCGGTGGAAATCCGGAACTGTCCCGCAACGGTATGGT

              P5                             P5'            B12-box        P1'
SCO nrdA   TGCGTGCATCCGTACGTCTTCGCACGTGCGCGCACGCCTGTCCAGTCCGAGGACCTGCCG
SAV nrdA   TGTGCGTGTTCGCGTAT-TTCGC---TGCCACGTGCTCGCTTCAGTCCGAGGACCTGTCG
SSC nrdA   CGCGTGCGTTCATGCCC-TTC-------TGCGCACGCCTGTTCAGTCCGAACACCTGTCG
SLI nrdA   TCGCGCTCT---------TTC------------GCGCGGCCGGAGTCCGAAGACCTGTGG

            P1'
SCO nrdA   ACAGTGCGCCCGG-CCGCCCTGGCCCGGGTGCCA-TGACGTCCGGGCCTCGCGGAGTGGG
SAV nrdA   ACAGCGCGCCCCGGCCGACC-GGCCCGGGTGCCA-TGACGTCCGGGCCTCGCGGAGTGGG
SSC nrdA   ACAGCGCGCCCGG-CCGTCC-GGTCCGGGTGCC--TGACGTCCGGGCCTCGTGGAATGGG
SLI nrdA   ACAGCGCACCCGGTCCGTCC-GTTCCGGGTGCCACAGACGTCCGGGCCTCGCGGAGTGGG

SCO nrdA   CCGGTGGACGCGACGCCGCGTGCGCTCGTGAGCTGCCCCCTGCCCTCCC-TCCGCCG---
SAV nrdA   CCGGTGGACGCGCGCGTGCCTGCCGTGCCGTGCGCCCTGCTGCCC-----TCCAACA---
SSC nrdA   CCGGTGGACGCGACGCCCCGTGCGCTCGTGAGCTGCCGCCTGCCC-----TCCGCCG---
SLI nrdA   CCGGTGGACGCGGCGCGCCGCGCGTATCCCGGCCCCCGCTCGTCCCCGTATCCCCCGTGC

                            RBS              fMet: NrdA translation →
SCO nrdA   -GGCCCCGTGCCGAGCGAGGGAGTGC-TCCACGTGACCATCGCGCCAGCCGATCCGGCTT
SAV nrdA   -GGCCCCGTGCCGAGCGAGGGAGAGCC-CCACGTGACCATCGCGCCAGCCGATCCGGCTT
SSC nrdA   -GGCCCCTTGCCGAGCGAGGGAGAGCC-CCACGTGACCATCGCGCCAGCCGACCCGGTTT
SLI nrdA   AGGCCACGAGCCGAGCGAGGGAGAGCCTCCCAGTGACCATCGCGCCCGCCGATCCGGCTT
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FIG. 1. (A) Chromosomal organization of the *S. coelicolor nrdABS* genes in the class Ia RNR operon. Gene designations: *nrdA* (SCO5226) and *nrdB* (SCO5225) encode the R1 and R2 subunits of the class Ia NrdAB ribonucleotide reductase, *nrdS* (SCO5224) encodes a putative AraC-like transcriptional regulatory protein, and SCO5228 encodes a putative GCN5-related *N*-acetyltransferase. Intergenic distances are given in numbers of base pairs, and arrows indicate direction of transcription of genes. (B) Nucleotide sequence alignment of *Streptomyces nrdABS* B12 riboswitches. Abbreviations: SCO, *S. coelicolor* M145; SAV, *S. avermitilis* MA-4680; SSC, *S. scabies* 87.22 (Sanger Centre); SLI, *S. lipmanii* NRRL 3584 (sequence from this work). The 123-nt B12 riboswitch is shown in bold letters. The 18-base B12 box is framed by an elongated dashed-line box. The P1, P3, P4, and P5 complementary stem-loop sequences are indicated by hollow arrows above the respective sequences and are labeled P1/1′, etc. Two solid arrows above the sequences indicate a partial inverted repeat, which may represent a transcriptional terminator. Two narrow boxes indicate complementary sequences present in the P4/P4′ riboswitch and in the putative terminator region. Other features shown in boldface italics are the −10 and −35 promoter recognition elements, the transcription start point (tsp), and the presumed ribosome binding sites (RBS) and GTG NrdA translational start codons.

GTG start codon (Fig. 1A) and led us to predict that it may play a role in regulating expression of the *nrdABS* operon (2). Sequence analysis of the *S. coelicolor* 5′-UTR revealed a putative 123-nt B12 riboswitch that is implicated in the regulation of B12-related genes in diverse bacteria (Fig. 1B). The consensus B12 riboswitch consists of a short conserved sequence domain called the B12 box and a set of base-paired stem-loops or complementary sequences, denoted P1/P1′, etc., distributed along the B12 riboswitch (14, 31). Its position immediately downstream of the promoter suggests that it functions as a sensor to regulate transcription. Similar B12 riboswitches were found in the 5′-UTRs of the corresponding *nrdABS* operons of *Streptomyces avermitilis*, *Streptomyces scabies*, and *Streptomyces lipmanii* (Fig. 1B). A partial inverted repeat immediately following the B12 riboswitch sequence is highly conserved in all four streptomycete sequences and may form a stem-loop structure that serves as a transcription

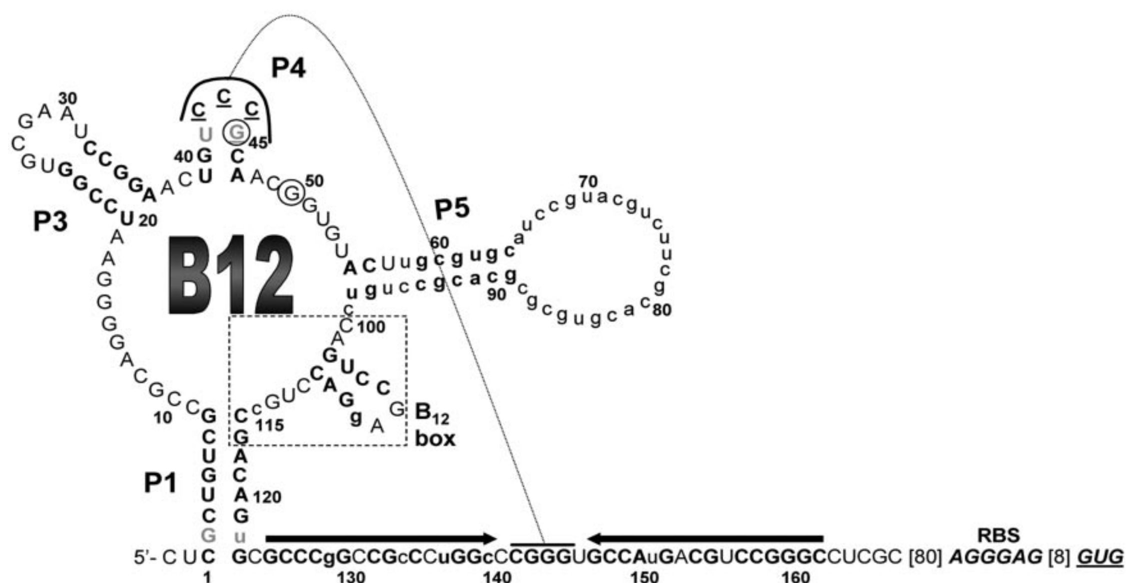

FIG. 2. Predicted secondary structure of the *S. coelicolor nrdABS* B12 riboswitch. The structure has been drawn according to the scheme presented by Vitreschak et al. (31). The conserved B12 box, the P1, P3, P4, and P5 complementary sequences, and the ribosome binding site (RBS) and GUG translation start codon are indicated. Two bold arrows show a putative transcription terminator region. Bases forming the B12 riboswitch are numbered 1 to 123, beginning at the stem of the P1 sequence. Numbers in brackets are bases outside the B12 riboswitch whose sequence is not shown. Bases that are fully conserved in the *S. coelicolor*, *S. avermitilis*, *S. scabies*, and *S. lipmanii nrdABS* B12 riboswitches are shown in uppercase letters, and nonconserved bases are shown in lowercase letters. AU and GC base pairs in the riboswitch and in the long inverted repeat following the riboswitch are shown in boldface letters, and UG base pairs are shown in lightface letters. The conserved bases CCCG in the P4 stem-loop are underlined and framed by a curved line. The complementary bases GGGC in the stem-loop of the putative transcription terminator are overlined. A hyphenated line indicates possible interaction between the P4 loop region and the loop region of the putative transcription terminator. In the absence of B12, the P4 loop region of the B12 riboswitch is postulated to interact with the complementary sequence in the loop region of the putative transcription terminator modifying and partly destabilizing its secondary structure and rendering it unable, or only partly able, to block transcription from the *nrdABS* promoter. B12 is thought to bind to, or near to, the P4 region of the riboswitch and to prevent interaction between the loop regions, enabling formation of a functional terminator that inhibits transcription. Two conserved guanines, G45 and G50, that were mutated and result in inhibition of B12 repression are circled. They are presumed to alter the secondary structure of the P4 region and to reduce B12 binding, enabling interaction between the loop regions, modifying the terminator, and rendering B12 ineffective in blocking transcription from the *nrdABS* promoter.

terminator. In *S. coelicolor*, the putative terminator sequence comprises a sequence of 16 nucleotides (14/16 identities) and a loop region, CCGGGU. Figure 2 shows the predicted secondary structure of the *S. coelicolor nrdABS* B12 riboswitch based on the secondary structure model of Vitreschak et al. (14, 30).

We searched the *S. coelicolor* genomic database for additional B12 riboswitches using as queries conserved sequences within the B12 riboswitches and identified similar sequences in the 5′-UTRs of eight other genes or gene clusters, most of which had been previously detected (http://rfam.wustl.edu/cgi-bin/getdesc?acc=RF00174; 14, 24), including *metE* encoding the B12-independent methionine synthase, *cobDQNXOBI* encoding B12 biosynthesis, *cbiM2NQ2O2* encoding cobalamin or cobalt transporting systems, *fedDC-btuC btuF-ftrDE* encoding iron transport systems and two ORFs of unknown function (Table 1). An exhaustive search of the complete genomes of *S. coelicolor*, *S. avermitilis* and *S. scabies* and the public databases pulled out 22 putative B12 riboswitches. All contained the P1, P3, P4, and P5 stem-loop sequences and the B12 box but differed with respect to the presence or absence of one or more of the P2, P6, and P7 stem-loops (Table 1) (unpublished data).

Rodionov et al. (24) reported that bacteria that possess genes encoding various B12-dependent systems were found to contain an alternative B12-independent system (but the opposite was not necessarily the case). We analyzed some 400 bacterial genomes for the presence of putative B12 riboswitch-like sequences near to class I and class III RNR genes and operons. We noted that whenever a B12 riboswitch was predicted to occur in front of class I and class III RNR genes we always detected in the genome genes encoding a class II RNR system. These bacteria have in common both a B12-dependent class II RNR (NrdJ) and a B12-independent class I RNR (NrdAB, NrdEF) or class III RNR (NrdDG) that are likely to be regulated by B12 riboswitches. A multiple alignment of putative B12 riboswitches of class I and class III RNR genes in gram-positive and gram-negative bacteria revealed high conservation of the P4/P4′ stem-loop region and the B12 box (see the supplemental material).

B12 inhibits growth of an NrdJ mutant lacking class II RNR activity. To assess the effect of B12 on the expression of the class Ia RNR, spores of M145 (wild type), M145Δ*nrdJ*, M145Δ*nrdJcobN*, and the control strains M145Δ*nrdB* and M145*cobN* were inoculated in medium containing or lacking B12, and growth was monitored according to optical density. M145Δ*nrdB* and M145Δ*nrdJ* contain an apramycin resistance cassette in place of *nrdB* and *nrdJ* abolishing the class Ia and II RNR systems, respectively; M145*cobN* contains a transposon insertion in the *cobN* gene that abolishes biosynthesis of B12,

TABLE 1. *Streptomyces coelicolor* A3(2) genes with B12 riboswitches

Locus tag	Gene/operon	Riboswitch coordinates[a]	Riboswitch stem-loops	Product/function/comments
SCO0985	*metE*	1037864–1038061	P1–P7	B12-independent methionine synthase
SCO0989		1044446–1044595 (−)	No P2	Unknown; *S. avermitilis* ortholog SAV6243 lacks a B12 riboswitch
SCO0991-SCO0995	*pduX* operon	1045892–1046113	P1–P7	Unknown; SCO0991 is similar to *Salmonella enterica* PduX (COG4542, possible kinase); SCO0992 contains a TTA leucine codon
SCO0996-SCO0998	*btuF-ftrDE* operon	1051426–1051573	No P2	Putative iron transporter, one of two paralog systems with a B12 riboswitch
SCO1847-SCO1853	*cobDQNOBI* operon	1975658–1975753	No P2, P6, P7	Cobalamin biosynthesis
SCO2321-SCO2325		2491669–2491851	No P7	Unknown; SCO2323-SCO2324 similar to CbiQ-CbiO; possible cobalt transporter
SCO5226-SCO5224	*nrdABS* operon	5688282–5688404 (−)	No P2, P6, P7	Class Ia ribonucleotide reductase
SCO5961-SCO5958	*cbiMNQO* operon	6532183–6532345 (−)	No P2	Putative cobalt and/or cobalamin transporter; the paralog operon SCO3159-SCO3161 lacks a B12 riboswitch
SCO7216-SCO7218	*fecCD-orf* operon	8019974–8020071	No P2, P6, P7	Putative FecCD family iron transporter; one of two paralog systems with a B12 riboswitch

[a] The coordinates of B12 riboswitches from P1 to P1′ are from this work; strand orientation is plus unless otherwise noted as (−).

which is an essential cofactor for the class II RNR. The parent strain M145, M145Δ*nrdJ* and M145Δ*nrdJcobN*, and the control strains M145Δ*nrdB* and M145*cobN* grew equally well in MY9 B12-deficient solid medium (Fig. 3A) and in liquid medium depleted of B12 (data not shown), confirming previous findings that the class Ia RNR (or class II RNR) is sufficient for normal growth (2). We next tested the effect of B12 on the growth of the NrdJ mutant. B12 concentrations of 1 μg/liter and higher completely prevented growth of the NrdJ and NrdJCobN mutants but had no effect on M145 or the control CobN and NrdB mutants (Fig. 3A). M145Δ*nrdJ* colonies resistant to B12 inhibition of growth are discussed below.

To further explore the effect of B12 on the growth of M145Δ*nrdJ*, we inoculated a fixed number of pregerminated spores in YEME B12-deficient liquid medium supplemented with different concentrations of B12 and measured the optical density at 450 nm after 25 to 50 h of incubation at 30°C (Fig. 3B). In the absence of added B12, M145Δ*nrdJ* had an optical density of about 0.5 at the mid-exponential phase of growth. A B12 concentration of just 0.01 μg/liter significantly affected growth of M145Δ*nrdJ* while concentrations of 0.5 μg/liter and higher essentially prevented growth. As controls, B12 concentrations of up to 100 μg/liter (the highest concentration tested) had no noticeable effect on growth of M145. If B12 was added to a culture that had reached the mid-exponential phase of growth, as opposed to being added at the pregermination stage, there was no apparent inhibitory effect on growth (Fig. 3C). These experiments indicate that B12 represses expression of the *S. coelicolor* class Ia RNR system and that the effect is growth dependent.

B12 represses transcription of *nrdABS* genes. To determine whether B12 regulates transcription of the *nrdABS* genes, mid-exponential cultures of M145Δ*nrdJ* were treated with 0 and 50 μg/liter B12 for 30 min, and total RNA was prepared. Northern blots of the RNA were then hybridized with *nrdB* and control *hrdB* probes (Fig. 4, left). In the absence of B12, *nrdABS* transcripts (~4.9 kb) were readily detected in M145Δ*nrdJ*. In the presence of B12, *nrdABS* transcription was significantly reduced within 30 min. In contrast, the same B12 concentration had no significant effect on *hrdB* transcripts nor did it inhibit growth of M145Δ*nrdJ* (Fig. 3C), presumably due to synthesis of sufficient class Ia RNR (prior to treatment) to allow continued normal growth. Quantitative real-time PCR measurements of *nrdABS* transcripts in mid-exponential cultures of M145Δ*nrdJ* treated with 0 and 50 μg/liter B12 for 30 min, as described above, are presented in Table 2. Equal amounts (2 μg) of total RNA were hybridized with an *nrdB* reverse (antisense) primer and incubated with reverse transcriptase, and the cDNA obtained was amplified and quantified by quantitative PCR. Results are reported as the ratio of the amount of *nrdABS* cDNA present in the untreated culture to that present in the B12-treated culture. The data show that B12 causes a four- to fivefold decrease in transcription of *nrdABS*. In contrast, B12 had no significant effect on transcription of the control *hrdB* gene. These experiments demonstrate that B12 regulates transcription of the *nrdABS* genes.

Characterization of B12 riboswitch mutants. When M145Δ*nrdJ* spores were plated on MY9 B12-deficient agar plates supplemented with 1 to 100 μg/liter B12, colonies appeared with a frequency of $\sim 10^{-3}$. We suspected that the resistant colonies were the result of alterations either in the B12 riboswitch overriding B12 repression of *nrdABS* transcription or in B12 uptake systems. The high frequency of resistant colonies was presumed to be due to the presence in the spore preparations of mutants that had arisen in previous culturing (in the absence of added B12) and was a consequence of selective pressure by the endogenous B12. When single colonies of M145Δ*nrdJ* were examined as above, B12-resistant variants occurred at a frequency of $\sim 10^{-5}$. This result was confirmed by constructing an *nrdJ cobN* double mutant deficient in the class II RNR and in B12 biosynthesis, which gave rise to resistant variants with a frequency of 10^{-6} to 10^{-7}. The same frequency of mutants was obtained when adenosyl-

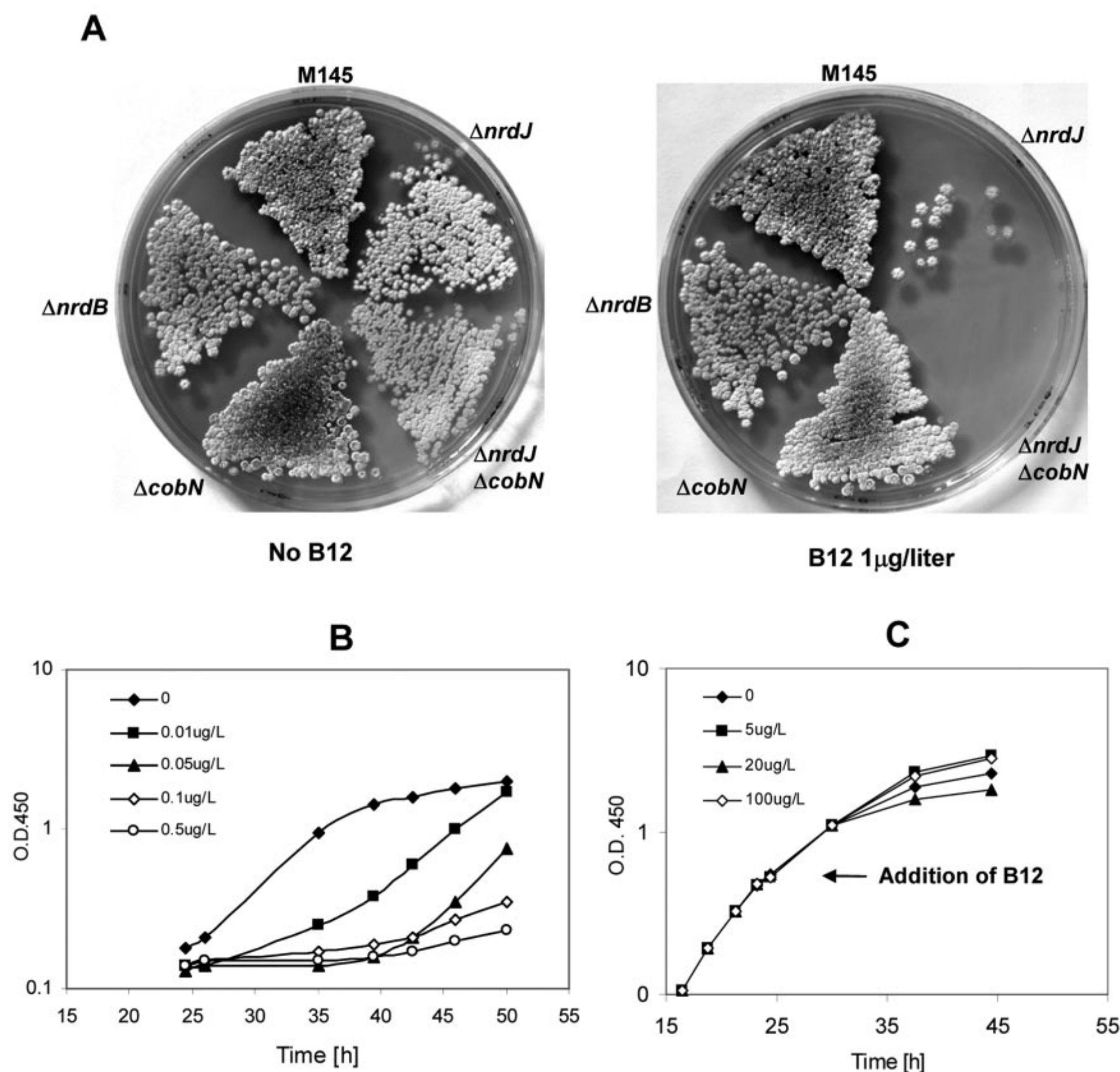

FIG. 3. Effect of B12 on growth of the class II NrdJ mutant. (A) Effect of B12 on growth of M145 (wild type), M145Δ*nrdJ*, M145Δ*nrdJcobN*, and control strains M145Δ*nrdB* and *M145 cobN*, in solid medium without B12 (left), or with addition of 1 μg/liter B12 (right). (B) Effect of B12 on growth of M145Δ*nrdJ* in liquid medium. Spores were inoculated in YEME medium (without antibiotics) containing B12 at a final concentration of 0.01, 0.05, 0.1, and 0.5 μg/liter, and growth at 30°C was monitored according to OD_{450}. (C) Cultures of M145Δ*nrdJ* were grown to mid-exponential phase, and B12 was added to a final concentration of 5, 20, and 100 μg/liter. Growth at 30°C was monitored according to OD_{450}.

cobalamin (coenzyme B12) was substituted with the analog cyanocobalamin (vitamin B12). Cyanocobalamin is converted to adenosylcobalamin in vivo, but in vitro it was reported to be ineffective in binding and in modulating translation initiation of *E. coli btuB* RNA (15, 16). Several B12-resistant colonies were chosen for study, and the individual clones were denoted by M145Δ*nrdJ* followed by a number. The isolates were indistinguishable in growth from M145Δ*nrdJ* (the parent strain), produced fluffy gray aerial mycelium, and sporulated well. All grew on MY9 plates containing B12 concentrations of up to 100 μg/liter (the highest concentration tested), whereas growth of M145Δ*nrdJ* was inhibited by 1 μg/liter. The mutants (and the parent strain) were unable to grow in the presence of 10 mM hydroxyurea (which inhibits class I RNRs), confirming their dependence for growth on a functional NrdAB RNR. Genomic DNA was extracted from 25 isolates, and PCR was used to amplify the ~600-bp DNA region encompassing the B12 riboswitch. Single-nucleotide substitutions were found in the DNA region encoding the B12 riboswitch in 11 of the 25 isolates. Two types of changes were found. In nine cases a guanine (G45) located in the P4 stem-loop was changed to a thymine (T); in two cases a second guanine (G50) located 5 nucleotides away on the 3′ side was changed to an adenine (A) (Fig. 2). Both mutations occur at sites that are completely or highly conserved in the B12 riboswitches of class Ia RNR operons in *Streptomyces* and in a variety of other bacteria (see the supplemental material). We isolated a further nine clones by employing lower B12 concentrations of 1 and 10 μg/liter. Two of the clones possessed the G45T mutation. Other mutations conferring B12 resistance were not mapped.

To assess the effect of the G45T and G50A mutations on transcription, RNA prepared after 30 min of B12 treatment of mid-exponential cultures of M145Δ*nrdJ* and M145Δ*nrdJ11* and

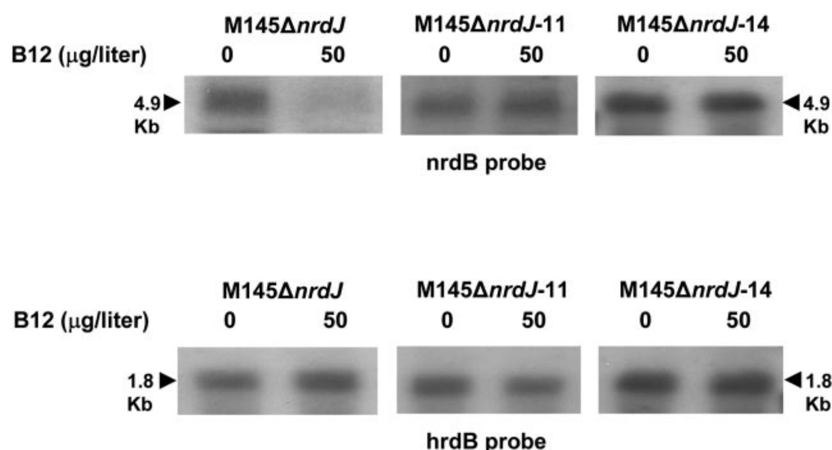

FIG. 4. Northern blot analysis of the *S. coelicolor nrdABS* genes in NrdJ and B12-resistant mutants. Total RNA from mid-exponential-phase cultures of M145Δ*nrdJ* and two B12-resistant mutants that had been treated with 0 and 50 μg/liter B12 for 30 min was hybridized with an *nrdB* probe. The positions of the ~4.9-kb *nrdABS* and ~1.8-kb *hrdB* mRNAs are shown by arrows. (Top) M145Δ*nrdJ* (left), M145Δ*nrdJ11* (center), and M145Δ*nrdJ14* (right) RNA hybridized with the *nrdB* probe. (Bottom) Corresponding hybridizations with the control *hrdB* probe. M145Δ*nrdJ11* and M145Δ*nrdJ14* carry G45A and G50T mutations, respectively, in the B12 riboswitch.

M145Δ*nrdJ14* containing the respective G45T and G50A mutations were hybridized on Northern blots to an *nrdB* probe and control *hrdB* probes (Fig. 4, center and right). B12 concentrations of 50 μg/liter had no discernible effect on transcription of *nrdABS* in either of the two mutant strains, whereas *nrdABS* transcription was much reduced in the parent strain M145Δ*nrdJ* (left). Thus, both classes of riboswitch mutations suppress the effect of B12 on inhibition of *nrdABS* transcription. These results were confirmed by quantitative PCR, which showed that B12 had no significant effect on transcription of *nrdABS* in either of the two mutant strains (Table 2).

DISCUSSION

Coenzyme B12 is an important cofactor in intermediary metabolism in bacteria, catalysis of intramolecular rearrangements, reduction of ribonucleotides to deoxyribonucleotides, and methylation. *Streptomyces* and some other bacteria contain two sets of RNR isozymes, one B12 dependent and another B12 independent. An analogous situation exists with respect to methionine synthetase. The MetH B12-dependent isozyme uses methylcobalamin (derived from B12) as a cofactor for transfer of the methyl group from N^5-methyltetrahydrofolate to homocysteine to form methionine. We are interested in understanding the individual roles of these alternative metabolic systems and the mechanisms by which they are regulated. In this work we have focused on B12 regulation of the class Ia RNR genes. Previously, we showed that in *Streptomyces* the *nrdABS* genes are transcribed in vegetative growth at a much lower level than the *nrdRJ* genes and that they are significantly upregulated when *nrdJ* is deleted or in a CobN mutant that is unable to synthesize B12. In this paper we show that B12 regulates transcription of *nrdABS* by a riboswitch mechanism.

Comparative genomics reveals that B12 riboswitches are widespread in bacteria (15, 24). Multiple sequence alignments of B12 riboswitches and other control elements indicate two main classes of RNA secondary structures. In one model, the effector molecule induces a stem-loop structure in the leader RNA that can then sequester the ribosomal binding site of the downstream gene and inhibit translation. In a second model, which is in accord with the results presented here, the effector molecule promotes formation of a stem-loop structure in the leader RNA, causing ρ-independent termination of transcription. Computer-predicted structures of B12 riboswitches suggest that in gram-negative proteobacteria translational initiation is the target of inhibition, whereas in the *Bacillus*/*Clostridium* group of gram-positive bacteria termination of transcription is indicated (14, 18, 30, 31). Premature termination of transcription by small metabolite molecules has been verified in experimental studies in *Bacillus subtilis* (14).

The nine *S. coelicolor* B12 riboswitches (Table 1) all contain a consensus B12 box and the P1, P3, P4, and P5 stem-loops of the conserved B12 riboswitch. The predicted secondary structures differ with respect to the presence or absence of the P2, P6 and P7 stem loops. According to Vitreschak et al. (30), B12 riboswitches can be classified into types, BI and BII, based on the presence of a conserved stem-loop region called BII which includes P6 and P7. The *S. coelicolor nrdABS* B12 riboswitch and most of the other B12 riboswitches lack the P2, P6 and P7 structures (Table 1) whereas the *metE* (SCO0985) and *pduX*-like (SCO0991) B12 riboswitches have a full complement of stem-loops. Differences were also evident when an alternative

TABLE 2. Effect of B12 on transcription of *S. coelicolor* M145 class Ia *nrdABS* RNR genes[a]

Strain	Ratio of level of *nrdB* transcripts (minus B12/plus B12)	Ratio of level of *hrdB* transcripts (minus B12/plus B12)
M145Δ*nrdJ*	4.1	1.2
M145Δ*nrdJ11*	1.1	ND
M145Δ*nrdJ14*	1.2	ND

[a] Numbers are the average values for three experiments using two independent RNA preparations. M145Δ*nrdJ11* and M145Δ*nrdJ14* contain G45A and G50T riboswitch mutations, respectively. ND, not determined.

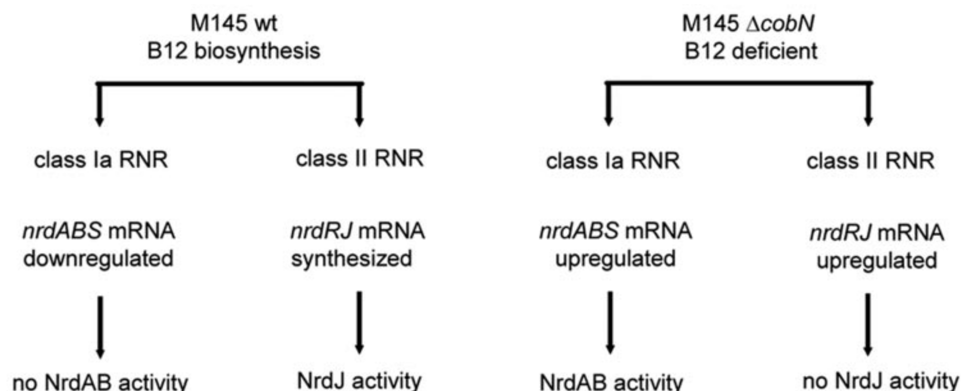

FIG. 5. Reciprocal effects of coenzyme B12 on controlling *Streptomyces coelicolor* class Ia and class II RNR systems. In M145 (wild type), B12 functions as a cofactor for the class II NrdJ RNR and represses transcription of the class Ia *nrdABS* RNR genes via a riboswitch. In the absence of B12 (in the CobN mutant), class II NrdJ RNR activity is abolished, and class Ia *nrdABS* RNR genes are relieved of repression. The mechanism of upregulation of transcription of class II RNR genes in the CobN strain is unknown.

scheme was used to represent the secondary structures (14). However, the function of the BII region is unknown. Streptomycetes contain, in addition to B12 riboswitches, other riboswitches that regulate thiamine, methionine, and flavin mononucleotide biosynthesis and the response to osmotic shock (http://www.sanger.ac.uk/Software/Rfam/).

B12 controls transcription of both sets of *Streptomyces* RNR genes. In an earlier study we showed that when B12 synthesis was abolished (in the CobN mutant strain) transcription of *nrdABS* was increased by about 30-fold (2), and we surmised that the effect was likely to be the result of eliminating B12 riboswitch repression. The experiments described here prove this to be the case. We explored the effect of B12 on transcription of the *nrdABS* genes by using a strain that expresses only the class Ia RNR. In solid medium M145Δ*nrdJ* was unable to grow in the presence of B12 concentrations as low as 1 μg/liter. Evidently, *S. coelicolor* imports B12 from the medium, possibly employing either one of the two putative cobalamin transporter systems (Table 1) to inhibit *nrdABS* transcription. In liquid medium, the same B12 concentration severely inhibited growth when present during spore germination but had little effect on growth when added to exponentially growing cells. Presumably, they contain enough of the class Ia RNR to support ongoing DNA synthesis, thereby masking the inhibitory effect of B12 on transcription. These and previous findings show that B12 controls RNR activity in two fundamentally different ways. In M145, the endogenous B12 is sufficiently high in concentration to (i) repress the class Ia RNR *nrdABS* riboswitch and (ii) provide enough cofactor to enable a fully functional class II RNR. In contrast, if B12 synthesis is abolished or falls below a threshold level, riboswitch repression of transcription of *nrdABS* is relieved, permitting expression of the class Ia RNR *nrdABS* operon and concomitantly eliminating or greatly reducing class II RNR activity. The finding that *nrdABS* transcription in M145Δ*nrdJ* is 10- to 20-fold higher than in M145 (2) supports the notion that the two RNR systems are interlocked and cross-regulated. Although we do not understand the mechanism that causes upregulation of *nrdABS* transcription in M145Δ*nrdJ*, we suppose that the increased number of *nrdABS* mRNA copies in the NrdJ mutant titrates out the endogenous intracellular B12 pool to override riboswitch control.

Figure 5 summarizes the reciprocal effects of B12 on the *Streptomyces* class Ia and class II RNR systems. B12 negatively controls expression of the class Ia RNR by a riboswitch mechanism and positively functions as an essential cofactor for the class II RNR. Elimination of the class II B12-dependent RNR system, the primary source of deoxyribonucleotides, by disrupting B12 synthesis or by eliminating NrdJ triggers increased expression of *nrdABS*. The effect of abolishing B12 on elevating transcription of *nrdRJ* appears to be indirect and due to a feedback mechanism. However, it cannot be simply due to lack of deoxyribonucleotides since in the absence of B12 the class Ia RNR genes are upregulated and produce sufficient enzyme to support normal growth. Elsewhere we have shown that NrdR (which is coexpressed with NrdJ) controls transcription of *nrdRJ* and *nrdABS*, probably by binding to repeat motifs located upstream of their promoter regions and repressing transcription (2). Consequently, NrdR and B12 both regulate, albeit in different ways, overall RNR activity. It seems likely that these two systems are poised to enable the balanced synthesis of deoxyribonucleotides in conditions where either one of the systems is not fully functional, for example, when one or more enzyme cofactors are limiting.

The above model predicts that mutations that alter the secondary structure of the *nrdABS* B12 riboswitch potentially inactivate the riboswitch and prevent B12 repression. Nahvi et al. (14, 15) have described two classes of mutants that affect binding of B12 to the 161-nt *E. coli btuB* B12 riboswitch, one modifying the conserved B12 box and another in the region termed P8. In the B12 RNA secondary structure scheme of Vitreschak et al. (30), the bases that form P8 correspond to the Add-II region but are absent in each of four *Streptomyces nrdABS* B12 riboswitches. We repeatedly identified two classes of mutations that mapped to a highly conserved portion of the B12 riboswitch. One mutation, G45U, maps to the P4 stem-loop; another, G50A, maps five nucleotides downstream (Fig. 2) (30). Alignment of 22 streptomycete B12 riboswitches showed that a G base is completely conserved at position 45, whereas a G or T base occurs at position 50. The consensus sequence for this region is $\mathbf{G_{45}C}$A/CG/A**C**G/**TGT**$_{52}$ (bold letters are fully conserved bases, and numbering is according to the *S. coelicolor nrdABS* B12 riboswitch) (Fig. 2) (7). In th revised structural model of the *E. coli btuB* riboswitch (14),

G45 and G50 correspond to G51 and G56 in the P5 and P6 sequences. The facts that G45T and G50A were the only mutations identified in the B12 riboswitch using B12 concentrations ranging from 1 to 100 μg/liter and that both map to the region of the P4 stem-loop, possibly altering its secondary structure, suggest that G45 and G50 are important for B12 binding. In the G45T and G50A mutant strains *nrdABS* transcription was unaffected by B12 concentrations as high as 100 μg/liter. A rationale for the effect of the mutations on overcoming B12 repression is based on the existence of complementary sequences in the P4 stem-loop region and the putative transcription terminator. In the absence of B12, the P4′ loop sequence—CCCG—potentially interacts with the complementary sequence in the loop region of the putative transcription terminator, destabilizing its secondary structure and thereby enabling transcription (Fig. 2). We suppose that B12 binds to the P4 stem-loop region to obviate this interaction, permitting a stable terminator stem-loop structure to be formed and preventing transcription. According to this model, the G45T and G50A mutations impede B12 binding, allowing interaction of the P4 mutant loop region with the transcription terminator, destabilizing its structure, and enabling constitutive transcription. Studies to test this model by in vitro measurements of B12 binding to wild type and mutant riboswitches are currently under way. A third class of B12-resistant mutants could not be mapped in the *nrdABS* 5′-UTR. They may confer resistance by blocking uptake of B12 and map to the predicted B12 riboswitches of two putative cobalamin transport operons, SCO2321-SCO2325 and SCO5961-SCO5958.

In conclusion, the studies reported here establish that the B12 riboswitch is an important control element in transcriptional regulation of the *Streptomyces* class Ia RNR genes. We note that B12 may function in a similar way to control the *S. coelicolor* B12-dependent and B12-independent methionine synthetases since the *S. coelicolor* (and *S. avermitilis* and *S. griseus*) *metE* gene encoding the B12-independent isozyme contains a B12 riboswitch in the 5′-UTR. The *Streptomyces* class II RNR is the primary RNR system in vegetative growth and functions to enable efficient growth recovery after oxygen deprivation. The class Ia RNR system may then function as a backup system when the class II RNR is inactive, for example, when B12 biosynthesis is limiting due to insufficient availability of cobalt or B12 biosynthetic precursors.

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