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Alternative oxygen-dependent and oxygen-independent ribonucleotide reductases in *Streptomyces*: cross-regulation and physiological role in response to oxygen limitation

Ilya Borovok,[1] Batia Gorovitz,[1] Michaela Yanku,[1] Rachel Schreiber,[1] Bertolf Gust,[2] Keith Chater,[2] Yair Aharonowitz[1] and Gerald Cohen[1]*

[1]*Department of Molecular Microbiology and Biotechnology, Tel Aviv University, Tel Aviv 69978, Israel.*
[2]*John Innes Centre, Norwich Research Park, Norwich NR4 7UH, UK.*

Summary

Ribonucleotide reductases (RNRs) catalyse the conversion of ribonucleotides to deoxyribonucleotides and are essential for *de novo* DNA synthesis and repair. *Streptomyces* spp. contain genes coding for two RNRs. We show here that the *Streptomyces coelicolor* M145 *nrdAB* genes encoding an oxygen-dependent class I RNR are co-transcribed with *nrdS*, which encodes an AraC-like regulatory protein. Likewise, the class II oxygen-independent RNR *nrdJ* gene forms an operon with a likely regulatory gene, *nrdR*, which encodes a protein possessing an ATP-cone domain like those present in the allosteric activity site of many class Ia RNRs. Deletions in *nrdB* and *nrdJ* had no discernible effect on growth individually, but abolition of both RNR systems, using hydroxyurea to inactivate the class Ia RNR (NrdAB) in the *nrdJ* deletion mutant, was lethal, establishing that *S. coelicolor* possesses just two functional RNR systems. The class II RNR (NrdJ) may function to provide a pool of deoxyribonucleotide precursors for DNA repair during oxygen limitation and/or for immediate growth after restoration of oxygen, as the *nrdJ* mutant was slower in growth recovery than the *nrdB* mutant or the parent strain. The class Ia and class II RNR genes show complex regulation. The *nrdRJ* genes were transcribed some five- to sixfold higher than the *nrdABS* genes in vegetative growth, but when *nrdJ* was deleted, *nrdABS* transcription was upregulated by 13-fold. In a reciprocal experiment, deletion of *nrdB* had little effect on *nrdRJ* transcription. Deletion of *nrdR* caused a dramatic increase in transcription of *nrdJ* and to a less extent *nrdABS*, whereas disruption of *cobN*, a gene required for synthesis of coenzyme B12 a cofactor for the class II RNR, caused similar upregulation of transcription of *nrdRJ* and *nrdABS*. In contrast, deletion of *nrdS* had no detectable effect on transcription of either set of RNR genes. These results establish the existence of control mechanisms that sense and regulate overall RNR gene expression.

Accepted 26 July, 2004. *For correspondence. E-mail coheng@post.tau.ac.il; Tel. (+972) 3640 9649; Fax (+972) 3642 2245.

Introduction

Ribonucleotide reductases (RNRs) provide the building blocks for DNA synthesis and repair in all living cells (Reichard, 1993). They are essential because they provide the only known *de novo* pathway for the biosynthesis of deoxyribonucleotides, the immediate precursors for DNA synthesis. Three major classes of RNRs are known. All use radical chemistry to create a protein thiyl-free radical that initiates reduction of ribonucleotides, and all use allosteric mechanisms to ensure the balanced formation of each of the four deoxyribonucleotides (Jordan and Reichard, 1998; Eklund *et al*., 2001). Class I RNRs are oxygen-dependent enzymes and are divided into two subclasses Ia and Ib. Class Ia enzymes occur in eukaryotes, most bacteria and some viruses. They consist of two homodimeric subunits. The larger catalytic subunit α_2 (R1) is encoded by *nrdA* and contains the active site, and substrate and allosteric effector binding sites. The smaller subunit β_2 (R2), encoded by *nrdB*, contains the cofactor binding sites. In the presence of molecular oxygen, it forms a stable di-iron tyrosyl-free radical. Class Ib RNRs are confined to bacteria and are distinguished from the Ia enzymes in that they lack ≈100 N-terminal residues and are not inhibited by dATP. Class II RNRs, encoded by *nrdJ* (Jordan and Reichard, 1998), are oxygen-independent enzymes that occur in aerobic and anaerobic bacteria in monomeric or oligomeric form. They use coenzyme B12 (adenosylcobalamin) to generate a transient 5′-deoxyadenosyl radical. The best characterized member of

this group, the *Lactobacillus leichmannii* enzyme, is a monomer of ≈82 kDa (Booker and Stubbe, 1993; Booker *et al*., 1994; Sintchak *et al*., 2002).

Despite significant differences in their primary sequence, subunit structure and cofactor usage, these enzymes and the class III RNRs that are confined to bacteria that grow in anaerobic conditions share a common three-dimensional core and possess similar reaction mechanisms (Reichard, 1997; Stubbe *et al*., 2001). This has led to the view that the three RNR classes possess a common origin and that ribonucleotide reduction is an ancient reaction that was a crucial link in the emergence of a DNA from an RNA world (Reichard, 1993; 2002; Torrents *et al*., 2002). The fact that many bacteria and archaea possess more than one class of RNR presumably reflects their evolutionary history and varied life cycles, whereas eukaryotes use just the class Ia RNR (Jordan and Reichard, 1998; Torrents *et al*., 2000; 2002; Poole *et al*., 2002). In some cases the rationale for having more than one RNR system is evident. In facultative aerobes such as *Staphylococcus aureus*, a class Ib RNR is used exclusively for aerobic growth whereas a class III RNR is limited to anaerobic growth (Masalha *et al*., 2001). In other bacteria, such as *Pseudomonas aeruginosa*, which contains genes coding for all three kinds of RNR, the situation is more complex (Jordan *et al*., 1999).

We recently reported that *Streptomyces* spp. contain class Ia and class II RNRs (Borovok *et al*., 2002). Streptomycetes are Gram-positive high G+C aerobic bacteria that belong to the actinomycetes, and have been intensively studied for the extraordinary variety of valuable metabolites they produce, and for their complex life cycle (Hopwood, 1988; Chater, 1993). The *Streptomyces* class Ia and class II RNR genes are differentially expressed during vegetative growth but their individual physiological roles are unknown. In this article, we describe a genetic approach for exploring this issue. We show that either one of the *Streptomyces coelicolor* class Ia and class II RNRs is sufficient for normal vegetative growth, that the class II RNR facilitates growth recovery after oxygen deprivation and that control mechanisms exist to integrate regulation of the RNR genes during vegetative growth.

Results

A functional class Ia or class II RNR is sufficient for S. coelicolor *vegetative growth*

Streptomyces spp. contain genes encoding class Ia and class II RNRs. *Streptomyces clavuligerus* class II RNR activity was readily detected whereas class Ia RNR activity was undetected. Consistent with this the class Ia RNR genes were transcribed in vegetative growth at a much lower level than the class II RNR genes (Borovok *et al*., 2002). To determine whether *S. coelicolor* can grow using the class Ia or the class II RNR, polymerase chain reaction (PCR)-targeted mutagenesis was used to replace the *nrdB* and *nrdJ* genes with a cassette containing the apramycin resistance gene (*apr*) (Gust *et al*., 2003). The growth profiles of M145 and the Δ*nrdB::apr* and Δ*nrdJ::apr* deletion mutant strains were indistinguishable in liquid (Fig. 1) or on solid medium (Fig. 2). *S. coelicolor* can therefore grow normally using either of its two RNR systems. We attempted to create a mutant deficient in both class Ia and class II RNRs by replacing *nrdB* in M145Δ*nrdJ::apr* with a cassette containing the streptomycin/spectinomycin resistance gene. Repeated trials failed to generate any authentic mutants implying that deletion of both genes is lethal and that *S. coelicolor* does not possess any other functional RNR systems (indeed, no other genes encoding RNR-like proteins are present in the complete genome sequences of *S. coelicolor* and *Streptomyces avermitilis*). Correlation of this conclusion was obtained from a study of the effect of hydroxyurea – a potent inhibitor of class I RNRs (Sinha and Snustad, 1972; Jordan *et al*., 1994) – on the growth of M145 and the *nrdB* and *nrdJ* deletion mutant strains. Figure 2 shows that all three strains, M145, M145Δ*nrdB::apr* and M145Δ*nrdJ::apr*, grew equally well on solid medium whereas the M145Δ*nrdJ::apr* strain alone failed to grow in the presence of 10 mM hydroxyurea. Quantitative measurements using spores and experiments performed in liquid medium supplemented with hydroxyurea supported this result (results not shown).

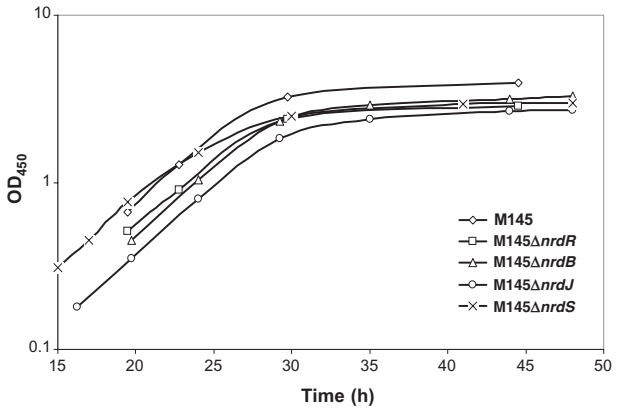

Fig. 1. Growth of *S. coelicolor* M145 and class Ia and class II RNR mutant strains in liquid medium. Cultures of M145 (wild type), M145Δ*nrdB* (M145Δ*nrdB*::*apr*), M145Δ*nrdJ* (M145Δ*nrdJ*::*apr*), M145Δ*nrdS* (M145Δ*nrdS*::*apr*) and M145Δ*nrdR* were grown in YEME medium (without antibiotics) at 30°C and the growth was monitored by optical density (OD_{450}).

Growth of S. coelicolor *after oxygen limitation is facilitated by the class II RNR*

Streptomycetes are considered to be strict aerobes that can survive for long periods in the absence of oxygen. In

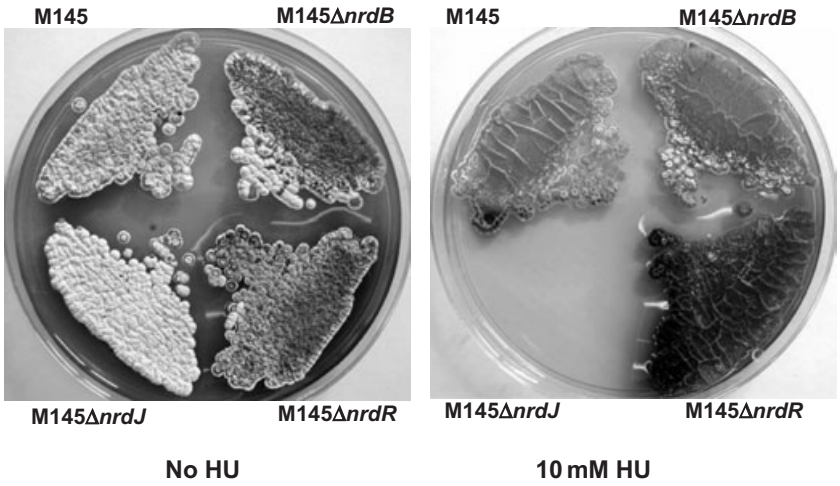

Fig. 2. Effect of hydroxyurea (HU) on growth of *S. coelicolor* M145 and class Ia and class II RNR mutant strains. Cultures of M145, M145Δ*nrdJ*, M145Δ*nrdB* and M145Δ*nrdR* were spread on MY9 plates supplemented with Middlebrook medium and incubated at 30°C with or without 10 mM HU. Strain designations as in legend to Fig. 1.

an attempt to define specific roles for the individual RNR systems, we examined the effect of mutations in the RNR systems on the ability of *S. coelicolor* to recover growth after oxygen starvation. Figure 3A shows the growth of M145, M145Δ*nrdJ::apr* and M145Δ*nrdB::apr* on solid medium after a period of 3 day incubation in an anaerobic chamber and subsequent exposure to aerobic conditions. No growth was visible on the plates during the anaerobic incubation. When the plates were exposed to air for 1 day (Fig. 3A, plate 2), M145 and M145Δ*nrdB::apr* consistently

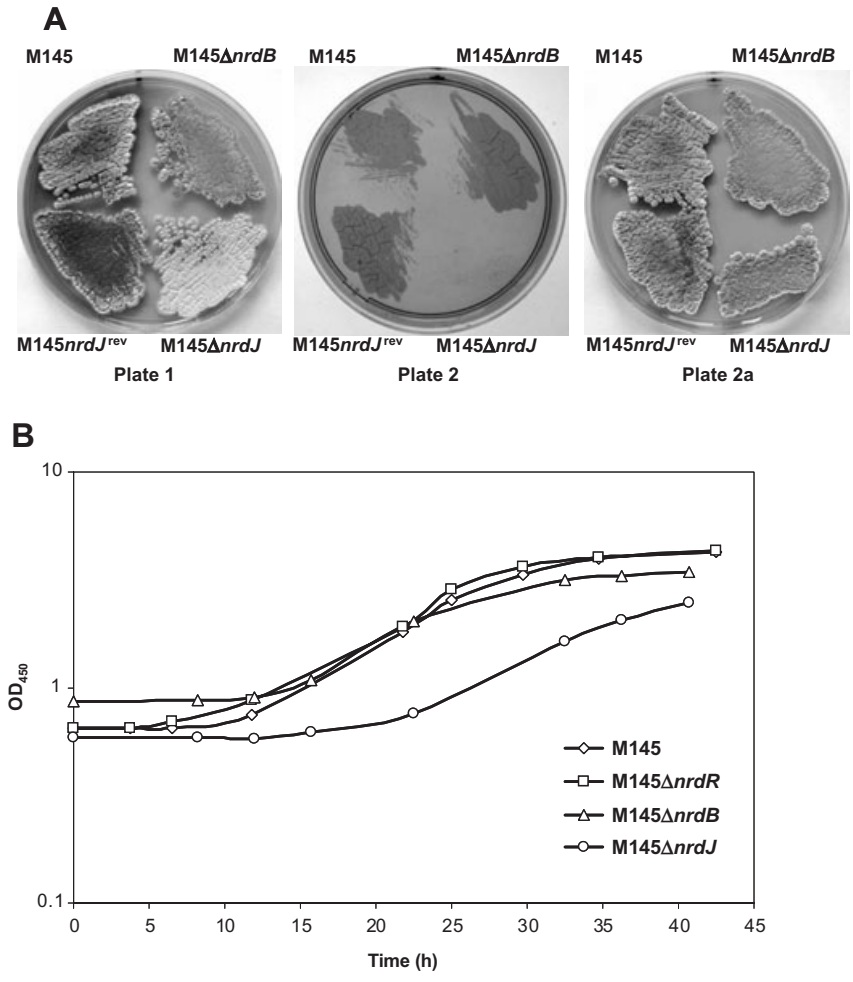

Fig. 3. Growth of M145 and class Ia and class II RNR mutant strains after incubation in anaerobic conditions and subsequent exposure to oxygen.
A. Solid medium. Cultures of M145, M145Δ*nrdJ*, M145Δ*nrdB* and M145*nrdJ*^{rev} were spread on MY9 plates supplemented with Middlebrook medium and incubated in an anaerobic chamber. Strain designations as in legend to Fig. 1; M145*nrdJ*^{rev} is a derivative of M145Δ*nrd::apr* in which the Δ*nrdJ*::*apr* deletion–substitution was replaced with the wild-type *nrdJ*$^{+}$ DNA region from cosmid 4H2. Conditions: plates 1 and 2 were incubated aerobically at 30°C for 24 h. Plate 1 was left to incubate aerobically whereas plate 2 was transferred to an anaerobic jar, incubated for 2–3 days and photographed after exposure to air for 1 day. Plate 2a is plate 2 photographed 4 days after completion of the anaerobic challenge.
B. Liquid medium. Early exponential aerobically grown cultures of M145, M145Δ*nrdJ::apr* and M145Δ*nrdB*::*apr* in YEME ($OD_{450} \approx 0.4–0.5$) were incubated in anaerobic conditions for 4 days at 30°C and growth was monitored after their transfer to aerobic conditions.

showed substantial growth whereas M145Δ*nrdJ::apr*, which lacks a functional oxygen-independent RNR, showed no detectable growth. Exposure of plates to air for a further 3 days (Fig. 3A, plate 2a) showed that M145Δ*nrdJ::apr* had recovered growth to the same extents as the parent M145. The same result was obtained when the experiment was repeated using spores; the appearance of M145Δ*nrdJ::apr* colonies after transfer of plates from the anaerobic chamber to aerobic conditions was significantly slower than that of M145 and M145Δ*nrdB::apr* (results not shown). When the apramycin cassette in M145Δ*nrdJ::apr* was replaced with the wild-type *nrdJ* gene (M145*nrdJ*^{rev}) the growth lag was abolished and growth was indistinguishable from that of the parent strain M145. Figure 3B shows that similar results were obtained in experiments performed in liquid culture. The *nrdJ* mutant strain exhibited a pronounced lag in growth after anaerobic incubation.

The S. coelicolor *class Ia and class II RNR transcription units contain structural and regulatory genes*

The nrdJ *transcription unit.* In a previous study, we noted that an open reading frame, termed *orfR*, is located immediately upstream of the *S. clavuligerus* and *S. coelicolor nrdJ* genes, and that close homologues of *orfR* are conserved throughout the eubacteria, although absent from archaea (Borovok *et al*., 2002). Northern blot analysis of *S. coelicolor* total RNA using probes specific for each of the genes showed that *orfR* and *nrdJ* are expressed as a single mRNA of ≈3.7 kb, which is the expected size if the two genes are co-transcribed (Fig. 4A; see below). We have therefore renamed *orfR* as *nrdR*. In *S. coelicolor*, the *nrdR* ATG translational codon is positioned 583 bp downstream of the *lexA* GTG start codon and the interval between *nrdR* and *nrdJ* is 168 bp. In streptomycetes, in *Propionbacterium acnes* (GenBank CQ363792) and in *Thermobifida fuscaYX* (formerly considered a streptomycete: DOE Joint Genome Institute database), *nrdR* is invariably flanked by *lexA* and *nrdJ* whereas in other actinomycetes, such as *Mycobacterium tuberculosis*, *Corynebacterium glutamicum* and *Bifidobacterium longum*, *nrdR* and *nrdJ* are unlinked. In A+T-rich Gram-positive bacteria *nrdR* is often just upstream of genes involved in DNA metabolism (e.g. *dnaB* and *dnaI*), whereas in the γ-proteobacteria *nrdR* is in the riboflavin biosynthetic operon and is called *ribX.* It has been tentatively proposed as a putative pathway regulator (see Kasai and Sumimoto, 2002).

In streptomycetes, the *nrdRJ* genes are divergently transcribed at distances of several hundred base pairs from the upstream *lexA* gene. S1 and reverse transcription polymerase chain reaction (RT-PCR) analysis of *S. coelicolor* total RNA identified a single transcription start point about 60–65 nt upstream of the *nrdR* ATG start codon (data not shown). Comparison of the corresponding DNA region upstream of the *Streptomyces jumonjinenesis*, *Streptomyces lipmanii*, *S. clavuligerus* and *S. avermitilis nrdR* genes revealed the likely positions of their promoter regions (Fig. 4B) and the presence of two conserved

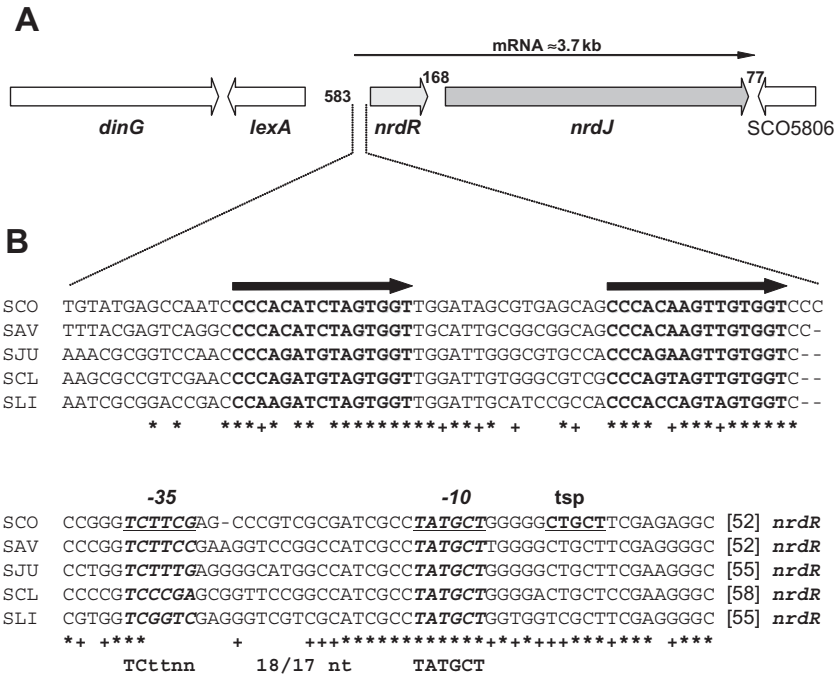

Fig. 4. Chromosomal organization of the *Streptomyces* class II RNR genes and sequence of the promoter region.
A. Organization of genes. Gene designations: *nrdJ* (SCO5805) encodes the class II NrdJ B_{12}-dependent ribonucleotide reductase, *nrdR* (SCO5804) encodes a transcriptional regulatory protein, *lexA* (SCO5803) encodes the LexA global regulator protein, *dinG* (SCO5802) encodes an ATP-dependent helicase, SCO5806 encodes a putative stress response protein. Intergenic distances are given in numbers of base pairs (bp). Arrows indicate direction of transcription.
B. Nucleotide sequence alignment of the promoter region upstream of *S. coelicolor* (SCO) *nrdRJ* with that of *S. avermitilis* (SAV), *S. jumonjinenesis* (SJU), *S. clavuligerus* (SCL) and *S. lipmanii* (SLI) *nrdRJ.* The positions of the *S. coelicolor* transcription start site (tsp) and −10 sequence motif are shown; two conserved tandem 15 bp direct repeats, located at 105–110 nucleotides upstream of the *nrdR* translational start codon, are indicated by bold arrows: asterisks indicate identical nucleotides in all five sequences, + indicate four-fifths of the nucleotides are identical. Numbers in brackets are number of nucleotides from the end of the shown sequence to the downstream transalational start codon.

A

Zn ribbon domain

MH**C**PF**C**RHPDSRVVDSRTTDDGTSI**RRRR**Q**C**PD**C**SRRFTTVETCS
ccccccccccceeeeceeccccceeeeeeccccccccccecchc

ATP-cone domain

LMVVKRSGVTEPFSRTKVINGVRKACQGRPVTEDALAQLGQRVEE
eeeeeccccccceceeeeeeceeeccccccchhhhhhhhhhhhh

AVRATGSAELTTHDVGLAILGPLQELDLVAYLRFASVYRAFDSLE
hhhhhchhhhehhhhhhhhhchhhhhhhhhhhhhhhhhhhhhhhh

acidic domain

DFEAAIAELRETTGHPGEEDDTGAGSQENDRGPTGAGQVPEPAGAAD
hhhhhhhhhhhccccccccccccccceeccccccccccccccchhh

B

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NrdA_EC LLVTKRDGSTERINLDKIHRVLDWAAEGLHNVSISQVELRSHIQF
NrdR_SC LMVVKRSGVTEPFSRTKVINGVRKACQGRPVTEDALAQL-GQ-RV
        *:*.**.* ** :.  *: . :  *.:*   .. : .:* .: :.

NrdA_EC YDGIKTSDIHETIIKAAADLISRDAPDYQYLA-ARLAIFHLRKKA
NrdR_SC EEAVRATGSAELTTHDVGLAILGPLQELDLVAYLRFASVYRAFDS
         :.::::.  *   : ..  *     : : :*  *:* .:   .:

NrdA EC YGQFEPPALYDHV
NrdR SC LEDFEAAIAELRE
          :**..    :
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Fig. 5. Predicted amino acid sequence, domains and secondary structure of the *S. coelicolor* NrdR protein.
A. The NrdR protein possesses three domains, an N-terminal region containing two pairs of cysteines (shown in bold) that may participate in forming a Zn ribbon-like fold, a long central domain possessing an ATP-cone motif (shown boxed) that is present in the N-terminal region of certain class I and class III RNRs, and a C-terminal acidic domain of unknown function. The predicted secondary structure is displayed beneath the sequence: h, helical; e, extended sheet; c, coil.
B. Sequence alignment of the N-terminal segments of the *E. coli* NrdA (R1) and *S. coelicolor* NrdR proteins. Residues in the *E. coli* R1 subunit that contact ATP in the allosteric site and are conserved in NrdR are shown boxed; other conserved residues are shown as identical (*), strongly similar (:) or weakly similar (.).

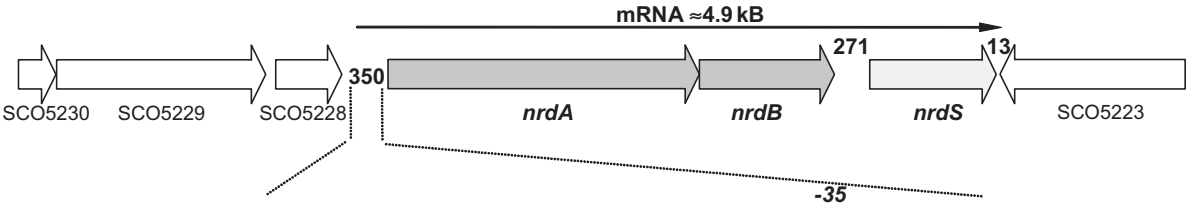
15 bp direct repeats. A search of the *S. coelicolor* genome found some 15 such sequences, containing 12 or more identities out of 15, with more than half of the sequences being located in regions upstream of genes encoding regulatory proteins, including representatives of the TetR, MoxR and MarR families.

The *nrdR* gene codes for a 182-amino-acid hydrophilic protein with an isoelectric point of 5.17 (Fig. 5A). NrdR contains in the N-terminal portion two pairs of vicinal cysteines that resemble the active-site domain found in glutaredoxin, thioredoxin and other small thiol redox proteins, a long central portion predicted to contain an ATP-cone that is found in the allosteric effector site of certain class I and class III ribonucleotide reductases (Aravind *et al*., 2000) and a C-terminal acidic domain. A multiple alignment of some 140 NrdR orthologues showed that the cysteines in the N-terminal portion are all conserved, as is a sequence of four consecutive arginines (data not shown). The existence of an ATP-cone domain in the *S. clavuligerus* NrdR protein was predicted in a systematic search (Aravind *et al*., 2000) of the non-redundant protein databases using the PSI-BLAST program (Altschul *et al*., 1997) using different RNRs as queries. The authors suggested that the ATP-cone can combine with the N-terminal cysteines to form a four-cysteine rubredoxin-like Zn ribbon module that functions as a transcriptional regulator. Comparison of the sequences of the putative NrdR ATP-cone domain with that present in the *Escherichia coli* NrdA protein, for which a three-dimensional structure exists (Eriksson *et al*., 1997), and other NrdR proteins, shows that they share significant sequence similarity and that the four residues that form hydrogen bonds with ATP are conserved (Fig. 5B).

The nrdABS *transcription unit.* The *S. coelicolor* class Ia RNR operon contains three genes, *nrdABS* (Fig. 6A). Northern blot analysis of total RNA using probes specific for each of the genes showed that they are expressed as a single mRNA of ≈4.9 kb which is the expected size if all three genes are co-transcribed (see below). The *nrdAB* genes overlap and encode the large and small subunits of the class Ia RNR; *nrdS* is located 271 bp downstream of *nrdAB.* In *S. avermitilis*, the *nrdAB–nrdS* interval is much larger (≈900 bp) and the region is annotated as encoding a 169-amino-acid unknown protein (SAV3028). The predicted *S. coelicolor*, *S. avermitilis* and *S. lipmanii* (GenBank AJ295339) NrdA proteins lack the N-terminal region present in the *E. coli* class Ia RNR that specifies the allosteric effector activity site for binding ATP and dATP. In this respect NrdA protein resembles the bacterial class Ib NrdE protein that also lacks this site (Jordan and Reichard, 1998).

S1 and RT-PCR analysis of *S. coelicolor* total RNA identified a single transcription start site about 300 nt upstream of the *nrdA* GTG start codon (data not shown) (Fig. 6A). Previously we proposed that the transcribed upstream region codes for a hypothetical 91-amino-acid ORF (SCO5227), but comparison of the sequence data for the corresponding regions in *S. lipmanii*, from this work (AJ295339), and that for *S. avermitilis* from the recently published genome sequence, reveals numerous differences from the *S. coelicolor* sequence that rule out the existence of this ORF (data not shown). A more detailed analysis of the long 5′ untranslated leader sequence of *nrdABS* mRNA revealed a conserved structural element that is involved in the regulation of B12-related genes in bacteria (Vitreschak *et al*., 2003). We noted the presence of such B12 structural elements in the corresponding regions upstream of the *S. lipmanii* and *S. avermitilis nrdABS* genes, the *cobD* gene (SC01847) in the adenosylcobalamin biosynthesis *cobDQN* gene cluster and the B12-independent methionione synthase gene, *metE* (SCO0985). Similar sequences were also noted in front of gene clusters for a putative cobalt transporter and two

A

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                                                                -35
S. coelicolor  CACCGTCACGGCACCGCGCCGGGACACAACATCTGGGGGTGCTCGCGTCCCCCGG
S. avermitilis AACCTCTCCGGCAC--CGACGGGACACAAGATGTGGGGGTGCCACCGGGGCCCGG
                ***    ******  ** ********** ** *********   **    *****
                                                          GCnnnC
                          -10          tsp
S. coelicolor  CACAAGATGTATGCTCATGCTCGCTG-[256 bp including B12-element]-nrdA
S. avermitilis CACAAGATGTATGCTCATGCTCGCTG-[248 bp including B12-element]-nrdA
S. lipmanii    nnCAACATGTATGCTCGTCCTCGCTG-[242 bp including B12-element]-nrdA
                 *** ********** * *****
                        TATGCT
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B

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NrdS_SC    MLRN--------------------VAAVLLDGAHPFELGVVCEVFGIDRSDEGLPVYDFA
AdpA_SC    MSHDSTAAPEAAARKLSGRRRKEIVAVLLFSGGPIFESSIPLSVFGIDRQDAGVPRYRLL
AdpA_SG    MSQDSAAATE-AARKLTGRRRREVVAVLLFSGGPIFESSIPLSVFGIDRQDAGVPRYRLL
           * ::                    **.:*:.*.  ** .:  .******.* *:* * :

NrdS_SC    VVSAEGPTLGTHVGGLTVSTPYGLERLEEADLIAVPAGSDFVRREYPPDLLDALRRATDR
AdpA_SC    VCAGEDGPLRT-TGGLELTAPQGLEAISRAGTVVVPAWRSITS-PPPEEALDALRRAHEE
AdpA_SG    VCGGEEGPLRT-TGGLELTAPYGLEAISRAGTVVVPAWRSITS-PPPAEALDALRRAHEE
           * ..*  .* * .*** :::* *** :..*. :.***  .:.    * : ******* :.

NrdS_SC    GTRVLSVCSGVFVLGAAGLLDGRQCAVHWHHAAELARQHPRARVAPDVLYVDEDPVVTSA
AdpA_SC    GARIVGLCTGAFVLAAAGLLDGRPATTHWMYAPTLAKRYPSVHVDPRELFVDDGDVLTSA
AdpA_SG    GARIVGLCTGAFVLAAAGLLDGRPATTHWMYAPTLAKRYPSVHVDPRELFVDDGDVLTSA
           *:*::.:*:*.***.******** .:.** :*. **:::* .:* *  *:**:. *:***

NrdS_SC    GTAAGIDACLHIVRKEQGPEVANRIARRMVVPPHRDGGQAQYIERPLPRSS-CDTVGEVL
AdpA_SC    GTAAGIDLCLHIVRTDHGNEAAGALARRLVVPPRRSGGQERYLDRSLPEEIGADPLAEVV
AdpA_SG    GTAAGIDLCLHIVRTDHGTEAAGALARRLVVPPRRSGGQERYLDRSLPEEIGSDPLAEVV
           ******* ******.::* *.*. :***:****:*.*** :*::*.**..  .*.:.**:
                          1st HTH domain
NrdS_SC    AWMEQHLDEEVTVEQLAVRAHMSPRTFARRFQQETGTTPYRWILRQRVLLAQRLLEATDE
AdpA_SC    AWALEHLHEQFDVETLAARAYMSRRTFDRRFRSLTGSAPLQWLITQRVLQAQRLLETSDY
AdpA_SG    AWALEHLHEQFDVETLAARAYMSRRTFDRRFRSLTGSAPLQWLITQRVLQAQRLLETSDY
           **  :**.*:. ** **.**:** *** ***:. **::* :*:: **** ******::*
                    2nd HTH domain
NrdS_SC    TMDTIAWRAGFGTAAALRHQFTRALDTTPHAYRRTFRG--PEAAA---------------  322 (of 322)
AdpA_SC    SVDEVAGRCGFRSPVALRGHFRRQLGSSPAAYRAAYRARRPQGDRQPDPDTAAAGATRPL  358 (of 398)
AdpA_SG    SVDEVAGRCGFRSPVALRGHFRRQLGSSPAAYRAAYRARRPQGVAESAATVVETMVPSQG  357 (of 405)
           ::* :* *.** :..*** :* * *.::* *** ::*.  *:.
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NrdS_SC/AdpA_SG: Identical aa **45.4%**; Strongly similar aa **17.2%**; Weakly similar aa **13.5%**
NrdS_SC/AdpA_SC: Identical aa **44.5%**; Strongly similar aa **17. 6%**; Weakly similar aa **12.5%**

Fig. 6. A. Chromosomal organization of the *S. coelicolor nrdABS* genes in the class Ia RNR operon. Gene designations: *nrdA* (SCO5226) and *nrdB* (SCO5225) encodes the large R1 and small R2 subunits of the class Ia NrdAB ribonucleotide reductase, *nrdS* (SCO5224) encodes a putative AraC-like transcriptional regulatory protein; other genes, SCO5230 encodes an unknown protein, SCO5229 encodes a putative praline transporter protein, SCO5228 encodes a putative GNAT-like acetyltransferase, SCO5223 encodes a putative P450 cytochrome. Intergenic distances are given in numbers of base pairs (bp), broad arrows indicate direction of transcription of genes.
B. Sequence alignment of the *S. coelicolor* NrdS protein with the *S. griseus* AdpA protein (AB023785) and the *S. coelicolor* AdpA orthologue (SCO2792). Two helix–turn–helix motifs (HTHs) in the C-terminal domain that are characteristic of the AraC family of proteins are shown underlined; two conserved cysteines are shown boxed. See legend to Fig. 5 for notation regarding other conserved residues. *S. coelicolor* NrdS shares 44.5% and 45.4% identity with *S. coelicolor* AdpA and *S. griseus* AdpA respectively.

iron transporters (SCO5961, and SCO0996 and SCO7216 respectively).

The *S. coelicolor nrdS* gene codes for a 322-amino-acid protein belonging to the large AraC family of transcriptional regulators (Fig. 6B). It is predicted to possess an N-terminal amidase domain and two C-terminal helix–turn–helix (HTH) motifs (Gallegos *et al*., 1997; Rhee *et al.* 1998; Martin and Rosner, 2001). A search of the *S. coelicolor* genome using *nrdS* as the query revealed 10 ORFs sharing 48–34% sequence identity. One, SCO2792, is a close homologue of the *Streptomyces griseus* AdpA transcriptional regulator (84.4% identity between the gene products) (Ohnishi *et al*., 1999; Horinouchi, 2002) and has recently been shown to be necessary for normal *S. coelicolor* aerial mycelium formation: it is the *bldH* gene (Nguyen *et al*., 2003; Takano *et al*., 2003). A second, SCO7512, is a close homologue of the *Streptomyces aureofaciens* GapR regulator (89% identity between the gene products) (Sprusansky *et al*., 2001). The other homologues have no known function. Most of these AraC-like proteins contain two conserved cysteines, located at positions 140 and 201 (*S. coelicolor* NrdS numbering) which are absent in the *E. coli* AraC protein, as illustrated in Fig. 6B, which shows an alignment of NrdS with the AdpA proteins of *S. griseus* and *S. coelicolor*. NrdS shares about 45% identity with both AdpA proteins.

Complex effects of nrdB*,* nrdJ *and* cobN *mutations on transcription of class Ia and class II RNR genes*

To determine whether the *nrdRJ* and *nrdABS* operons are differentially transcribed, and to assess the effect of deletions of the RNR-encoding genes on transcription, Northern blots of total RNA from early exponential cultures of M145, M145Δ*nrdB::apr* and M145Δ*nrdJ::apr* were hybridized with *nrdR and nrdJ* probes (Fig. 7A) and *nrdA*, *nrdB* and *nrdS* probes (Fig. 7B). *nrdRJ* transcripts (≈3.7 kb) in the parental strain M145 were most prominent in young cultures with the level progressively decreasing with the age of the culture (data not shown). Deletion of *nrdB* in M145Δ*nrdB::apr* had a marginal effect on *nrdJ* transcription (Fig. 7A, lanes 1 and 2, left and middle). The transcripts were perceptibly more abundant in strain KF61 containing a *cobN* mutation that abolishes synthesis of B12 (adenosylcobalamin), an essential cofactor for the class II RNR (Fig. 7A, lanes 4, left and middle). As a control, the *hrdB* gene was found to be transcribed at essentially the same level in M145, M145Δ*nrdB::apr*, M145Δ*nrdJ::apr* and KF61*cobN::*Tn*4560* (Fig. 7A, right). In contrast to these results, we were unable to detect *nrdA*, *nrdB* and *nrdS* transcripts in young cultures of M145 (Fig. 7B, lanes 1, left, middle and right) or at later times (data not shown). However, *nrdABS* transcripts (≈4.9 kb) were readily detected in the *nrdJ* deletion mutant (Fig. 7B, lanes 3, left, middle and right). Transcription of the *nrdABS* genes was similarly elevated in the *cobN* mutant strain (Fig. 7B, lanes 4, left, middle and right). Northern blot results were confirmed and quantified by real-time RT-PCR experiments (Table 1) and confirm and extend earlier studies in *S. clavuligerus* which showed that the class Ia and class II RNR genes are differentially transcribed in vegetative growth (Borovok *et al*., 2002). We tested the effect of addition of B12 on transcription of *nrdABS* and *nrdRJ* in the KF61*cobN::*Tn*4560* strain. At B12 concentrations of 10 μg l^{-1} *nrdABS* and *nrdRJ* transcription was markedly reduced to levels similar to that in M145 (results not shown).

Deletion of nrdR *upregulates both* nrdRJ *and* nrdABS *transcription*

Streptomyces coelicolor M145 strains were constructed with deletions of the *nrdR* and *nrdS* genes. M145Δ*nrdS::apr* contains an apramycin resistance cassette in place of the *nrdS* gene and M145Δ*nrdR* was obtained after excising the cassette in M145Δ*nrdR::apr* leaving a 81 bp scar (see *Methods*). Both mutants exhibited normal growth in liquid and solid media and in the presence of hydroxyurea establishing that *nrdS* and *nrdR* are not essential for growth (Fig. 2). Moreover, *nrdR* is not essential for expression of *nrdJ* with which it is co-transcribed.

To further assess the effect of the *nrdR* deletion on transcription of the *nrdRJ* and *nrdABS* operons, Northern blots of total RNA from early exponential cultures of M145 and M145Δ*nrdR* were hybridized with *nrdA*, *nrdB* and *nrdJ*

Table 1. Copy numbers of class Ia and class II RNR transcripts in exponential cultures of M145 (wild-type) and mutant strains.

	Copy number[a]			
	M145	M145D*nrdB*	M145D*nrdJ*	M145*cobN*$^-$
nrdB probe (class Ia)	1.1×10^3	0.2×10^3	16.8×10^3	33.8×10^3
Induction ratio	1	0.18	15.3	30.7
nrdJ probe (class II)	5.4×10^3	6.8×10^3	0.54×10^3	52.4×10^3
Induction ratio	1	1.25	0.1	9.7

a. Copy numbers of cDNA per 67 ng of total RNA are the average values for three experiments using two independent RNA preparations.

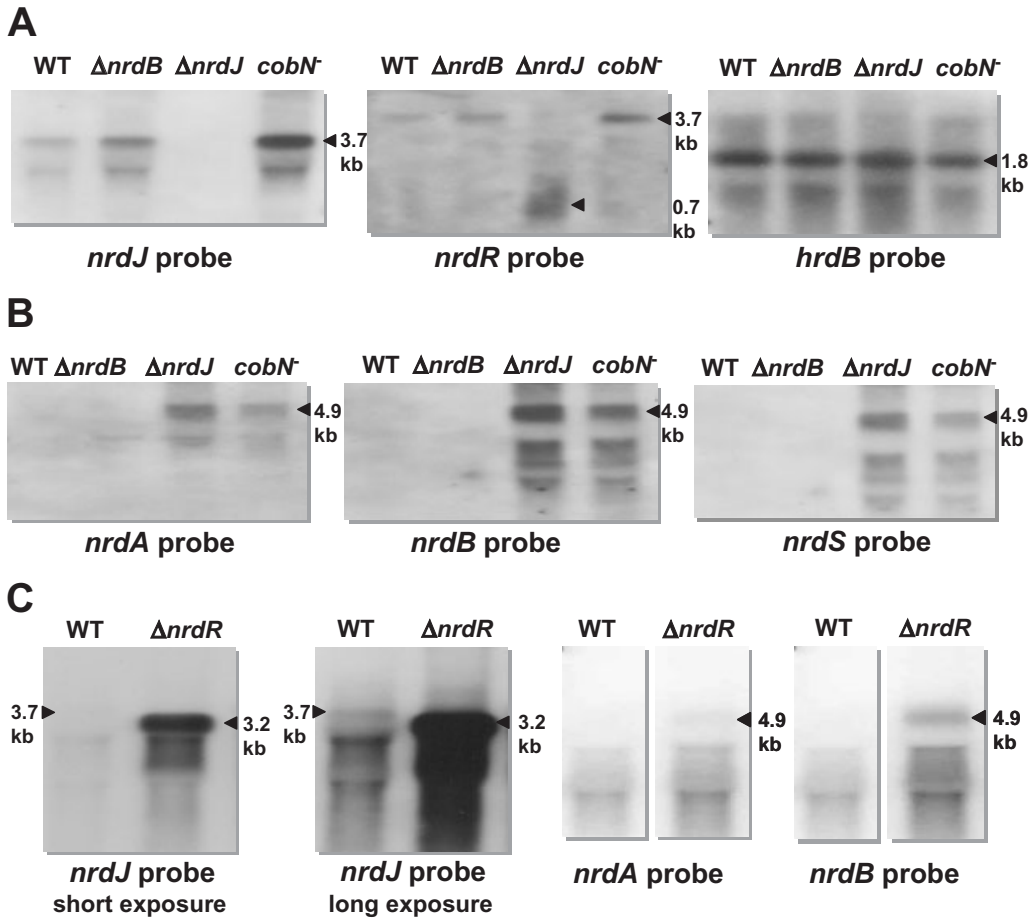

Fig. 7. Northern blot analysis of the *S. coelicolor* class Ia and class II RNR genes.
A. Effect of *nrdB*, *nrdJ* and *cobN* mutations on transcription of class II RNR genes. Total RNA from early exponential cultures of M145 (wild type), M145Δ*nrdB*::*apr* (Δ*nrdB*), M145Δ*nrdJ*::*apr* (Δ*nrdJ*) and KF61 *cobN*::TN*4560* (*cobN*$^-$) was hybridized with probes for: *nrdJ* (left), *nrdR* (middle) and the control *hrdB* (right). The positions of the ≈3.7 kb *nrdRJ* mRNA, ≈0.7 kb *nrdR* mRNA and the control ≈1.8 kb *hrdB* mRNAs are shown by arrows.
B. Effect of *nrdB*, *nrdJ* and *cobN* mutations on transcription of class Ia RNR genes. Total RNA from early exponential cultures of M145 (wild type), M145Δ*nrdB*::*apr* (Δ*nrdB*), M145Δ*nrdJ*::*apr* (Δ*nrdJ*) and KF61 *cobN*::Tn*4560* (*cobN*$^-$) was hybridized with probes for: *nrdA* (left), *nrdB* (middle) and *nrdS* (right). The position of the ≈4.9 kb *nrdABS* mRNA is shown by an arrow.
C. Effect of an in-frame *nrdR* deletion mutation on transcription of class Ia and class II RNR genes. Total RNA from early exponential cultures of M145 (wild type) and M145Δ*nrdR* (Δ*nrdR*) was hybridized with probes for: *nrdJ* (left and left middle), *nrdA* (right middle) and *nrdB* (right). The positions of the ≈3.2 kb *nrdJ* mRNA, the ≈3.7 kb *nrdRJ* mRNA and the ≈4.9 kb *nrdABS* mRNA are shown by arrows.

probes (Fig. 7C). In M145Δ*nrdR*, the *nrdJ* probe detected a ≈3.2 kb mRNA that corresponded in size to that expected for the partially deleted *nrdRJ* transcript (Fig. 7C, lane 2, left). The 3.2 kb transcript was more than 20-fold more abundant than the ≈3.7 kb *nrdRJ* mRNA made in M145 (Fig. 7C, lane 1, left) as estimated from a comparison of band intensities in different film exposures. These results indicate that NrdR negatively regulates transcription of the *nrdRJ* operon. Unexpectedly, deletion of *nrdR* also upregulated transcription of the *nrdABS* genes. As stated above, *nrdABS* transcripts could not be detected in M145; however, a ≈4.9 kb *nrdABS* mRNA was readily detected by *nrdA* and *nrdB* probes in M145Δ*nrdR* (Fig. 7C, lanes 2, middle and right). The effect of the *nrdR* in frame deletion was similar to that of the *nrdJ* deletion in strain M145Δ*nrdJ::apr* (Fig. 7B, lanes 3, left and middle). In contrast to the above results, deletion of *nrdS* (in M145Δ*nrdS::apr*) had no appreciable effect on transcription of *nrdRJ* or *nrdAB* genes (data not shown).

Discussion

The studies in this article were prompted by the finding that streptomycetes, strict aerobes, contain genes coding for oxygen-dependent and oxygen-independent RNRs. We show that: (i) class Ia *nrdAB* and class II *nrdJ* RNR genes are individually sufficient for growth and occur in operons containing genes encoding putative regulatory

proteins, (ii) *nrdJ* stimulates recovery of growth of *Streptomyces* after oxygen limitation and (iii) *nrdRJ* and *nrdABS* are differentially transcribed and regulated, expression of both gene sets being influenced by *nrdR*. These observations attest to the existence of control mechanisms that determine the overall expression of RNR genes.

NrdR is a transcriptional regulator of class Ia and class II RNR genes

The NrdR protein regulates transcription of both class Ia and class II RNR genes. Deletion of *nrdR* dramatically increased transcription of the *nrdRJ* operon and had a pronounced effect on transcription of the *nrdABS* operon. Thus, NrdR regulates its own synthesis. Plausibly, NrdR binds to the two 15 bp direct repeats in the DNA region upstream of its promoter and blocks transcription (Fig. 4B). Similar 15 bp sequences were found upstream of at least eight *S. coelicolor* genes, several of which themselves encode transcription regulators, so that NrdR might indirectly regulate a larger number of genes. Unexpectedly, NrdR also negatively regulates transcription of the *nrdABS* genes implying that expression of both class Ia and class II RNR genes is linked (see below). As there is no obvious potential NrdR-binding sequence in the *nrdABS* promoter region, its repression by NrdR may well be indirect. Alternatively, the 15 bp repeats may not be NrdR targets.

NrdR contains an ATP-binding domain that resembles that present in the allosteric effector activity site of certain ribonucleotide reductases. Class Ia RNRs contain within the α_2-subunit an allosteric effector activity site for binding ATP/dATP as well as a specificity site for binding ATP and dNTPs. The former site functions to regulate overall enzyme activity; when ATP is bound to that site enzyme activity is stimulated, when dATP is bound enzyme activity is inhibited (Jordan and Reichard, 1998). The crystal structure of the *E. coli* class I NrdA (R1) subunit shows that the effector activity site is located in the N-terminal region (Uhlin and Eklund, 1994; Eriksson *et al*., 1997). In other RNRs, for example, the prokaryotic class Ib and class II RNRs, including the *Streptomyces* class II RNR, this site is absent. It is of interest therefore that the *Streptomyces* class II RNR is activated by ATP and dATP (Borovok *et al*., 2002) and is coexpressed with NrdR. The regulatory significance of these findings is not yet clear, but it is tempting to consider the possibility that NrdR, together with ATP or dATP, may interact with NrdJ RNR to modulate its activity as well as acting as a transcriptional regulator.

NrdS is an AraC-like regulatory protein

NrdS is a member of the AraC family of bacterial transcriptional regulators that either activate or repress promoters of diverse targets, to which they bind via two HTH motifs (Niland *et al*., 1996). The best characterized of the *Streptomyces* AraC-like proteins is AdpA, a master regulator of differentiation and in *S. griseus* of secondary metabolism (Horinouchi, 2002; Chater and Horinouchi, 2003; Takano *et al*., 2003). Another AraC-like protein, the *S. aureofaciens* GapR protein, regulates expression of the *gap* gene encoding glyceraldehyde-3-phosphate dehydrogenase (Sprusansky *et al*., 2001). A BLAST search revealed 10 open reading frames encoding *nrdS*-like genes in *S. coelicolor*, and 11 in *S. avermitilis.* With the exception of AdpA (SCO2792) and GapR (SCO7512), the functions of these AdpA/NrdS-like proteins are unknown. Most of the *S. coelicolor* and *S. avermitilis* AraC-like proteins contain two conserved cysteines which are absent in the *E. coli* and *Bacillus subtilis* AraC proteins and which may conceivably serve as redox sensors of oxidative stress. Another difference is the presence in the N-terminal portion of NrdS of a putative amidase domain. This feature serves to distinguish between the classic *E. coli* AraC family, designated COG2207, and a new family of regulatory proteins which includes NrdS and AdpA, designated COG4977 (Tatusov *et al*., 1997). A search of the *Mycobacteria* genome databases failed to identify any NrdS/AdpA/GapR homologues in these actinomycetes (with the possible exception of *Mycobacteria smegmatis* where two AdpA paralogs are predicted), a finding that may relate to the very different life cycles of the *Mycobacterium* and the *Streptomyces*. As AdpA is a regulator of development, the similar features present in NrdS and many other AraC-like proteins may indicate that it and AdpA both respond to some aspect of cellular physiology that is a signal for development and that NrdAB then plays a role specific to the resulting developmental state.

Complex regulation of class Ia and class II RNR genes

In a previous study, we showed that in *S. clavuligerus* class Ia RNR activity could not be detected in cell extracts whereas class II RNR activity was readily detected, and that the corresponding genes were transcribed at very different levels in vegetative growth (Borovok *et al*., 2002). Here we show that the *S. coelicolor* class Ia RNR genes are also differentially transcribed and that an *nrdJ* mutation that abolishes the class II RNR causes a greater than 10-fold increase of transcription of the class Ia RNR genes to a level comparable to that of the class II RNR genes in the parental strain. As the mutation had no discernible effect on growth, the class Ia RNR is evidently able to effectively substitute for loss of the class II RNR. In contrast, deletion of *nrdB*, which abolishes the class Ia RNR, had little effect on transcription of the class II RNR genes. Hence, the existence in *Streptomyces* of two RNR sys-

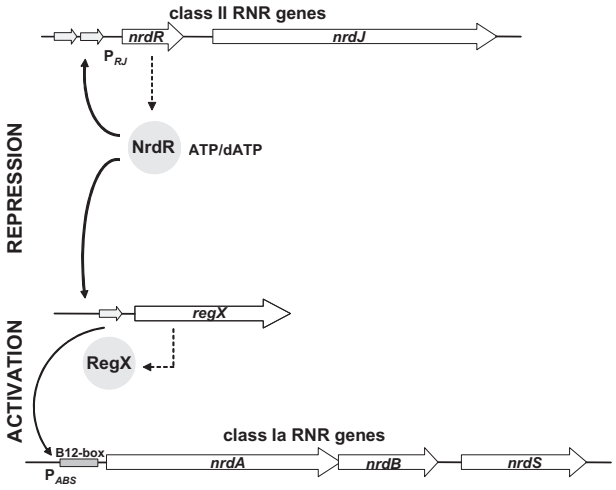

Fig. 8. A model for the role of NrdR in controlling transcription of the class Ia and class II RNR genes in *S. coelicolor*. NrdR directly negatively regulates transcription of the class II RNR genes from the promoter p_{RJ}, possibly by binding to a nearby tandem repeat sequence. Indirectly it downregulates transcription of class Ia RNR genes from the promoter p_{ABS} by blocking transcription of a hypothetical gene *regX* that encodes a transcriptional activator. Modulation of NrdR activity may occur by binding ATP or dATP. See text for other details.

tems ensures that a sufficient supply of deoxyribonucleotides is available for DNA synthesis in conditions in which one or other of the RNRs is limiting or not active. Ordinarily, the class II RNR genes are expressed at a high level and provide an adequate pool of DNA precursors for vegetative growth. Presumably, when the class II RNR is absent a feedback mechanism is activated that senses and responds to the depleted deoxyribonucleotide pool and upregulates transcription of the class Ia RNR genes. While the nature of this mechanism is unknown, it functions to integrate regulation of the class Ia and class II RNR genes. This view is borne out by the effects of *nrdR* and *cobN* mutations on transcription of both sets of RNR genes.

In the first place, deletion of *nrdR* resulted in a marked increase in transcription of the *nrdJ* gene. Transcription of *nrdABS* was also elevated. Plausibly, NrdR binds to specific sequences near to the *nrdRJ* promoter region and thereby prevents access of RNA polymerase, or partly inhibits a factor needed for transcription (Figs 4B and 8). As stated above, NrdR may regulate the expression of a number of other genes, referred to as target genes, several of which are themselves transcriptional regulators. However, we were unable to identify in the upstream region of the class Ia RNR genes any sequences similar to those present in the corresponding region of the class II RNR genes and the target genes. Possibly, one of the proteins encoded by the target genes, shown in the scheme as *regX*, is an activator of the class Ia RNR genes and its transcription is blocked by NrdR. A regulatory cascade of this type has been proposed to operate in the A-factor-dependent regulation of streptomycin synthesis in *S. griseus*. A-factor triggers release of a repressor ArpA which activates transcription of AdpA which in turn activates streptomycin biosynthesis (Ohnishi *et al*., 1999; Horinouchi, 2002). As NrdR is potentially able to bind ATP or dATP, these (and possibly other nucleotides) may modulate the proposed DNA binding activity of NrdR. Thus, in analogy with the A-factor regulatory cascade, the level of ATP/dATP in the cellular pool may provide the regulatory signal that controls the cascade. Studies are presently underway to determine whether the expression of any of the target genes is controlled by NrdR.

In the second place, a transposon insertion in the *cobN* gene had a qualitatively similar effect to that of the *nrdR* deletion in increasing transcription of the class Ia and class II RNR genes. C*obN* is located in a cluster of genes necessary for synthesis of coenzyme B12 (adenosylcobalamin), an essential cofactor for the class II RNR. In the wild-type strain the class II RNR is the major RNR used for synthesis of deoxyribonucleotides; in the *cobN* mutant strain (KF61) B12 synthesis is disrupted and class II RNR activity abolished. The effect of the *cobN* mutation on upregulating transcription of the class II RNR genes appears therefore to result from a feedback mechanism that senses the depleted deoxyribonucleotide pool and is perhaps similar to the mechanism invoked above for upregulating class Ia RNR genes in a class II RNR-deficient strain. Preliminary experiments show that B12 downregulates transcription of the class II RNR genes in the *cobN* mutant to the level in M145.

However, the effect of abolishing B12 synthesis on increasing transcription of the class Ia RNR genes may result from a rather different mechanism. Adenosylcobalamin is known to repress, primarily at the translational level, the expression of a variety of genes involved in B12 synthesis and transport and genes that use B12 as a cofactor (Richter-Dahlfors and Andersson, 1992; Nou and Kadner, 1998), by direct binding of B12 to a conserved RNA structural element, or B12-box, in the 5′ untranslated region of the gene (reviewed in Vitreschak *et al.* 2003). Rodionov *et al*. (2003) identified B12 elements in the 5′ untranslated regions of *S. coelicolor* genes determining B12-independent isozymes of methionine synthase and ribonucleotide reductase. We noted that the unusually long 5′ untranslated leader sequence of *nrdABS* contains a B12 structural element, and similar B12 structural elements are present in the upstream regulatory regions of several other *Streptomyces* genes including those of the *metE* B12-independent methionine synthase (SCO0985), *cobD* (SCO01847), the first gene in a large cluster of genes responsible for B12 synthesis, and the *cbiNMQO* (SCO5961/58), *fedDC* (SCO7216/17) and *ftrDE*

(SCO996/98) genes which are thought to be involved in cobalt and iron transport respectively. The elements are also present in the *S. avermitilis* orthologues. These findings suggest that upregulation of the class Ia RNR genes in the *cobN* mutant strain probably results from release of B12-dependent repression. Evidence to support this view comes from the finding that B12 abolishes growth of M145Δ*nrdJ* which lacks the class II RNR (I. Borovok, B. Gorovitz, M. Yanku, R. Schreiber, Y. Aharonowitz and G. Cohen, unpubl. results). Because B12 markedly reduces transcription of *nrdABS* genes in the *cobN* mutant, B12 represses expression of class Ia RNR genes primarily at the transcriptional level.

Survival of S. coelicolor *after oxygen deprivation is facilitated by the class II RNR*

The ideas presented in the above point to the potentially critical role that cofactor availability may have with regard to the activity of the two *Streptomyces* RNRs in their natural habitat. Depletion of cobalt would have a crippling effect on the functioning of the class II RNR system, whereas limitation of ferrous iron could have similar consequences for the class Ia RNR. *Streptomyces* may have therefore evolved two classes of ribonucleotide reductases to help cope with fluctuations in available cofactors. An additional cofactor of the class Ia RNR, oxygen, is considered below.

Class Ia and class II RNRs differ not only in the nature of their metallo-cofactors but also in another important way, in their requirement for oxygen; class Ia RNRs require molecular oxygen to create the protein radical necessary for enzyme activity whereas class II RNRs can function without oxygen. It is quite likely that *Streptomyces* spp., which are considered to be strict aerobes, encounter oxygen limitation at several different stages in their life cycle. In the soil, their natural environment, the level of oxygen a few centimetres below the surface may drop precipitously after heavy rain and water logging. In the laboratory, the amount of oxygen diffusing into a colony on an agar plate or within a dense mycelial culture falls off rapidly towards the interior. These considerations raise questions as to whether the *Streptomyces* class II RNR genes are expressed in limiting oxygen concentrations. This issue is currently under investigation in our laboratory and has recently been addressed in the related actinomycetes *M. tuberculosis* where it was demonstrated that the *nrdZ* gene, which potentially encodes the class II RNR, was upregulated in limiting oxygen (Dawes *et al*., 2003), perhaps enabling a low level of dNTP primarily for DNA repair during persistent infection.

The experiments reported here are consistent with this idea in that they show that abolishing this RNR results in a significant lag in growth after relief of oxygen deprivation. We suppose that the class II RNR can function at a low level under oxygen limitation and thereby provide a residual reservoir of deoxyribonucleotides for DNA repair and immediate use for DNA synthesis once oxygen limitation has been removed.

Experimental procedures

Bacterial strains, plasmids and growth conditions

Streptomyces coelicolor A3(2) strain M145 (referred to as wild type; Kieser *et al*., 2000) and derivative strains containing deletions in *nrd* genes (this study) were manipulated as described (Kieser *et al*., 2000). KF61 is M145 containing a Tn*4561* transposon insertion in the *cobN* gene (provided by T. Kieser). For routine subcloning, *E. coli* K-12 DH5α (Sambrook *et al*., 1989) and ET 12567 (*dam dcm hsdS*; MacNeil *et al.* 1992) were grown and transformed according to Sambrook *et al.* (1989). ET12567 was used to propagate unmethylated cosmid DNA for introduction into *S. coelicolor* by conjugation. *E. coli* BW25113/pIJ790 served as the host for λ-RED-mediated PCR-targeted mutagenesis (Gust *et al*., 2003), and *E. coli* DH5α/BT340 was the host for FLP recombinase-mediated deletion of disruption cassettes to leave non-polar, phenotypically unmarked deletion mutations (Datsenko and Wanner, 2000). pIJ773 (Gust *et al*., 2003) was used as the template for amplification of a disruption cassette containing *aac(3)IV* (conferring apramycin resistance) and the RK2 origin of transfer (*oriT*), flanked by recognition sites for FLP recombinase. *S. coelicolor* KF61 contains a Tn*4560* transposon insertion in the *cobN* gene.

Streptomyces media and culturing were as follows. MS agar (Kieser *et al*., 2000) was used to prepare spore suspensions and for plating out conjugations with *E. coli* ET12567 containing the RP4 derivative pUZ8002 (Flett *et al*., 1997); MY agar (Shirling and Gottlieb, 1966) supplemented with Middlebrook 7H9 Broth (4.7 g l^{-1}) (MY9) was used for growth of strains in anaerobic jars. YEME medium (Kieser *et al*., 2000) was used for liquid growth. Cultures of *Streptomyces* were obtained essentially as described (Strauch *et al*., 1991). When needed, media were supplemented with apramycin (Sigma) 50 μg ml^{-1}, viomycin (gift of T. Kieser) 30 μg ml^{-1} and hydroxyurea (Sigma) 10 mM. Adenosylcobalamin was from Sigma.

For incubation of *Streptomyces* in oxygen-limiting conditions, 20 μl of germinated spores were inoculated to 50 ml of YEME medium and grown for 20–25 h to OD_{450} 0.3–0.5. The culture was transferred to fill 15 ml tubes, sealed, and static incubation continued at 30°C for up to 90 h. For aerobic cultivation, the contents of the tube were transferred to a 100 ml flask containing glass beads and shaken at 250 r.p.m. at 30°C. Alternatively, *Streptomyces* cultures in MY9 medium, or spores suspensions, were spread on plates and incubated in a 2.5 l anaerobic jar (Oxoid) with an AnaeroGen sachet (Oxoid), or in a Bactron Anaerobic Chamber (Shel Laboratory).

Construction of S. coelicolor *nrd mutants*

Mutants in which the entire *nrdB*, *nrdJ*, *nrdR* and *nrdS* coding regions were deleted were constructed by PCR targeting

(Gust *et al*., 2003) using oligonucleotide primers with 5′ ends overlapping the 5′ and 3′ ends of the *nrd* coding sequences, and 3′ (priming) ends designed to amplify the apramycin resistance *aac(3)IV* disruption cassette of pIJ773: nrdB1 (5′-TCCCTGGAAAACCCCGAGTCCTGCGAGGCCTGCCAGTAAATTCCGGGGA TCCGTCGACC) and nrdB2 (5′-GGAGACCCGGACCCGCTACGGGAAGGAGACCC GGAGCACTGTAGGCTGGAGCTGCTTC); nrdJ1 (5′-GGGCACTTCAGGGCGTTTTCGCCCGTACAGGGAGGCGGCATTCCGGGGATCCGTCGACC) and nrdJ2 (5′-GGCGCCCCTTACTCATGGGCGGCTAGGAACGACTACGCGTGTAGGCTGGAGCTGCTTC); nrdR1 (5′-GGCCGTTGAGTCTGCTGTGAGGAGGGTTCGGAGTCCATGATTCCGGGGATCCGTCGACC) and nrdR2 (5′-GGGCCCGGGCGCCGAAGTCCGGGTCCGGCCCGCCGATCATGTAGGCTGGAGCTGCTTC); S1 (5′-GGCCTCGATTTCCTGCCACCAGCGAGGCACACTGGCGGCATTCCGGGGATCCGTCGACC) and nrdS2 (5′-TGCTGGTCAGGCCCGTGGCGCGGTGACGAC CCGGACGGGTGTAGGCTGGAGCTGCTTC).

The PCR product was introduced into *E. coli* BW25113/pIJ790 containing cosmid 4H2 (for *nrdR* and *nrdJ*) or cosmid 7E4 (for *nrdB* and *nrdS*), pre-induced for λ-RED functions by the addition of arabinose, to obtain the disrupted versions of the *nrd*-disrupted versions of the cosmids. The disrupted cosmids were isolated and transferred via *E. coli* strain ET12567/pUZ8002 to *S. coelicolor* M145 by conjugation. Single cross-overs were selected on MS agar containing apramycin. Colonies were replica plated and scored for kanamycin sensitivity. Deletions in *nrd* genes were confirmed by Southern hybridization using internal *nrd* DNA fragments amplified by PCR and labelled with PCR DIG Probe Synthesis kit (Roche). The M145Δ*nrd*::*apr* mutant strains were abbreviated M145Δ*nrdB*, M145Δ*nrdJ* and M145Δ*nrdS*.

To construct a phenotypically unmarked in frame *nrdR* deletion, the disrupted cosmid 4H2 *nrdR*::*aac(3)IV* was introduced into *E. coli* DH5α/BT340 to excise the disruption cassette from the cosmid by FLP recombinase. The resulting cosmid 4H2Δ*nrdR* was then transferred to *S. coelicolor* M145 by conjugation via ET12567. Single cross-over exconjugants were selected on MS agar containing kanamycin and apramycin. Several such colonies were taken through one round of non-selective growth on MS agar, and spores were plated for single colonies, which were scored for kanamycin and apramycin sensitivity. All the colonies tested were kanamycin and apramycin sensitive and were deleted for *nrdR.* Deletion of *nrdR* was confirmed by PCR and nucleotide sequencing. The *nrdR* in frame deletion strain was abbreviated M145Δ*nrdR*.

For complementation experiments, the apramycin resistance cassette in M145Δ*nrdJ*::*apr* was replaced with the wild-type *nrdJ* gene as follows: M145Δ*nrdJ::apr* was conjugated with the 4H2 cosmid (containing the wild-type *nrdJ* gene) in which the *bla* ampicillin resistance gene had been replaced with the *apr-oriT* cassette. Single cross-overs were selected for on MS agar containing kanamycin and apramycin. Colonies were replica plated and scored for kanamycin and apramycin sensitivity. The presence of an intact *nrdJ* gene was confirmed by PCR analysis and by the ability to grow in the presence of 10 mM hydroxyurea. The complemented strain is ostensibly identical to M145 and was designated M145*nrdJ*^{rev}.

Total RNA extraction

Total RNA was isolated from about 200 mg wet weight of exponentially growing cultures of *S. coelicolor* strains (OD_{450} 0.5–1) in YEME medium according to the modified Kirby procedure (Kieser *et al*., 2000). The TPNS reagent was replaced with *N*-lauryl sarcosine (Sigma L-9150). For RT-PCR experiments, RNA was treated with RQ1 RNase-free DNase (Promega), 10 U in 100 μl of total RNA in DEPC water, to remove residual DNA. RNA concentrations were determined by A_{260} measurements and RNA integrity was analysed by agarose/formaldehyde gel electrophoresis (Sambrook *et al*., 1989).

Northern blot analysis

Quantification of *S. coelicolor nrd* transcripts in total RNA was performed by Northern blot analysis as described (Masalha *et al*., 2001). RNA (5 μg) was electrophoresed in an agarose/formaldehyde gel and transferred to NytranN nylon membranes (Schleicher & Schuell). Internal *nrd* DNA fragments were amplified by PCR and labelled with the PCR DIG Probe Synthesis kit (Roche). The primers for PCR were: nrdJ6 (5′-CGCATGACCGGCGAGGTCAT) and nrdJ7 (5′-ATCAGCAGGGCGCCGAGGTT) producing a fragment of 415 bp; nrdA1 (5′-TGGATGACCTTCAAGGACAC) and nrdA2 (5′-AGCTGGAAGAAGACGTCCTG) producing a fragment of 344 bp; nrdB5 (5′-GGGACGAGACGATGCACATG) and nrdB6 (5′-GGGCGCCGCTCGAAGAAGTT) producing a fragment of 331 bp; nrdS3 (5′-TTCGAACTGGGCGTGGTCTG) and nrdS4 (5′-GTGCAGGCAGGCATCGATAC) producing a fragment of 467 bp; nrdR3 (5′-TGCGATTCGCCTCCGTCTAC) and nrdR4 (5′-GATCAGTCGGCGGCGCCT) producing a fragment of 186 bp. Control primers for monitoring *hrdB* transcripts were: hrdB1 (5′-CTCTGTCATGGCGCTCATTG) and hrdB2 (5′-AGGTAGTCCTTGACCGGGTC) producing a fragment of 605 bp.

RT-PCR

RT-PCRs to analyse co-transcription of *nrdRJ* and *nrdABS* were performed as described (Borovok *et al*., 2002) using AMV reverse transcriptase (Promega). Amplification was carried out in a T gradient Thermocycler f/96 (Biometra). Quantitative PCR was carried out in a LightCycler system (Roche Molecular Biochemicals) as described (Borovok *et al*., 2002) using the pairs of primers listed in the section on *Northern blot analysis*. Total RNA (2 μg) in a 20 μl reaction containing 1× AMV buffer (Promega), 1 mM dNTPs, 0.625 μM of reverse primer was denatured for 5 min at 80°C, 10 U of reverse transcriptase and 20 U of RNasin (Promega) were added and the mixture was incubated for 45 min at 57°C and heated for 5 min at 100°C. A 20 μl reaction mix containing 12.5 pmole each primer, 3.5 mM $MgCl_2$ and 5% DMSO and cDNA (2 μl) was dispensed into a sealed capillary tube, pre-incubated at 95°C for 10 min and subjected to 40 cycles of amplification, 15 s at 95°C (temperature transition 20°C s^{-1}), 10 s at 66–62°C (step size 0.4°C, step delay one cycle) for *nrdJ* cDNA or 60–55°C (step size 0.5°C, step delay one cycle) for *nrdJ* and *nrdB* cDNAs, and 18 s at 72°C (temperature transition

2°C s^{-1}). Detection of fluorescent DNA products was monitored once per cycle at 87°C. Melting curve analysis was performed in the range 70–98°C to confirm that a single DNA product was made with the same melting temperature as the control PCR standard DNA. Copy numbers of cDNAs were obtained from standard curves made with serially diluted samples of purified PCR products.

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.nim.nih.gov/gorf/.html) and the CLONE MANAGER 7 program (Scientific and Educational Software, Durham, NC). Primary sequences of putative class II RNRs, NrdS-like and NrdR-like proteins were identified in unannotated genomic databases via the NCBI site (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) by use of BLAST algorithms (BLASTp and tBLASTn) (Altschul *et al*., 1997). More detailed BLAST searches of *S. coelicolor* and *S. avermitilis* genomes were prepared in http://www.sanger.ac.uk/Projects/S_coelicolor/ and http://avermitilis.ls.kitasato-u.ac.jp/ respectively. Pairwise sequence alignments were prepared with the BLAST 2 SEQUENCES server in NCBI (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html) and multiple sequence alignments were made with the CLUSTAL W program, version 1.84 (Higgins *et al*., 1996) using the EMBL ClustalW server (http://www.ebi.ac.uk/clustalw/).

Nucleotide sequencing

Nucleotide sequencing was determined using an ABI Prism 3100 Genetic Analyser (Applied Biosystems) and the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems), as recommended by the manufacturers, except that 5% (v/v) DMSO was added to each reaction. Sequences were determined on both strands and submitted to the GenBank database (Accession No. AJ276618, AJ295339, AJ586904, AJ586905).

Other methods

Southern hybridization was carried as described (Kieser *et al*., 2000). Probes were made by labelling DNA fragments with DIG using the DNA labelling and detection kit (Roche).

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