

***Streptomyces* spp. contain class Ia and class II ribonucleotide reductases: expression analysis of the genes in vegetative growth**

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Genes encoding two ribonucleotide reductases (RNRs) were identified in members of the genus *Streptomyces*. One gene, *nrdJ*, encoded an oligomeric protein comprising four identical subunits each with a molecular mass of ~ 108 kDa. The activity of this protein depended on the presence of 5'-deoxyadenosylcobalamine (coenzyme B₁₂), establishing it as a class II RNR. The *Streptomyces clavuligerus nrdJ* gene was cloned, using internal peptide sequences from the purified protein, and was found to encode a polypeptide of 961 aa. Molecular phylogenetic analysis showed that the *S. clavuligerus* class II RNR shares significant similarity with most other bacterial and archaeal class II RNRs. Two other genes, *nrdA* and *nrdB*, were initially identified in the *Streptomyces coelicolor* genome database in unannotated ORFs as encoding a class Ia RNR. Southern analysis demonstrated that the *nrdAB* genes were present in different *Streptomyces* spp. The *S. coelicolor nrdAB* genes were cloned and expressed in *Escherichia coli*, and the recombinant proteins were shown to represent a class I RNR. It was shown, using quantitative real-time PCR, that the *S. clavuligerus* class Ia and class II RNR genes were differentially transcribed during vegetative growth. The copy number of the class II *nrdJ* transcripts was approximately constant throughout the exponential phase of vegetative growth ($3\text{--}5 \times 10^5$ copies per 400 ng total RNA after reverse transcription). In contrast, the copy number of the class Ia *nrdAB* transcripts was some 10- to 20-fold less than that of *nrdJ* in the early-exponential growth phase (2.8×10^4 copies), and decreased markedly at the mid-exponential (4×10^3 copies) and late-exponential phases (1.1×10^3 copies) of growth. A possible role for the involvement of two RNRs during vegetative growth is discussed.

Keywords: *Streptomyces*, 5'-deoxyadenosylcobalamine, expression, phylogeny, ribonucleotide reductase genes

INTRODUCTION

Streptomyces spp. are Gram-positive, filamentous soil bacteria that produce a remarkable variety of secondary metabolites (Hopwood, 1988; Horinouchi & Beppu,

1990) and have a complex cycle of morphological differentiation (Chater, 1993). During the last few years, it has emerged that the genus *Streptomyces* and many other members of the high-G + C branch of the actinomycetes, including the genus *Mycobacterium*, possess an unusual thiol redox metabolism, unusual in that they lack glutathione and its associated enzymes and contain millimolar concentrations of a novel low molecular mass glycothiol, termed mycothiol, and a broad-range thioredoxin system (Aharonowitz *et al.*, 1993; Newton *et al.*, 1993, 1995, 1996). Low molecular mass thiols, and the enzyme systems that operate on them, play a crucial

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Abbreviations: coenzyme B₁₂, 5'-deoxyadenosylcobalamine; RNR, ribonucleotide reductase.

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role in many cellular processes, chief among which are the protection of cells against toxic oxygen species, the maintenance of the intracellular thiol–disulphide redox status, and the provision of electrons to key reductive enzymes such as ribonucleotide reductase (RNR). All living organisms employ one or more RNRs to carry out the reduction of each of the four ribonucleotides to deoxyribonucleotides for DNA synthesis and repair (Reichard, 1993).

Several classes of RNRs have been described, which differ in their protein structure and in the nature of the protein radical in the catalytic site (Jordan & Reichard, 1998). In the microbial world it is not uncommon to find organisms that possess two, or even three, different RNRs (Jordan *et al.*, 1999). Class I RNRs have an $\alpha_2\beta_2$ -subunit structure and are present in all higher organisms, in certain viruses and in eubacteria, but are not present in archaea. They consist of two homodimeric proteins, R1 and R2. In class Ia RNRs, the R1 (α_2) protein is encoded by the *nrdA* gene and the R2 (β_2) protein is encoded by the *nrdB* gene. The larger α -chain contains the binding sites for substrates and effectors, and has five catalytic cysteines that are responsible for the reduction of all four ribonucleotides. The smaller β -chain contains an oxygen-linked diferric centre that in its active form has a stable tyrosyl free radical that removes the hydrogen atom from the 3' carbon of the nucleotide substrate. Class Ib RNRs are confined to certain eubacteria. They possess the same $\alpha_2\beta_2$ -subunit structure and metal centre as the class Ia enzymes (the corresponding R1 and R2 proteins are encoded by the *nrdE* and *nrdF* genes, respectively), but they share only modest sequence identity with class Ia RNRs and also differ in some functional aspects. All class I RNRs employ molecular oxygen in catalysis. The immediate source of the reducing power for class Ia enzymes comes from one of two small proteins, thioredoxin or glutaredoxin, each of which contains a pair of redox-active cysteines. Thioredoxin is maintained in its reduced form by thioredoxin reductase, whereas glutaredoxin is kept reduced by glutathione–glutathione reductase. In both cases the reduced state is maintained at the expense of NADPH (Holmgren, 1989). A glutaredoxin-like protein, NrdH, functions as a hydrogen donor to class Ib enzymes (Jordan *et al.*, 1996, 1997).

Class II RNRs are widespread among aerobic and anaerobic eubacteria and archaea, and they employ 5'-deoxyadenosylcobalamine (coenzyme B₁₂) as the radical generator in an oxygen-independent process. The best-characterized class II enzyme is that from *Lactobacillus leichmannii*, a monomeric protein of ~82 kDa that functions in an equivalent manner to protein R1 (Panagou *et al.*, 1972; Blakley, 1978; Booker & Stubbe, 1993). Comparison of the deduced amino acid sequence of the cloned *L. leichmannii* RNR gene, *nrdJ*, with class I RNRs shows that it contains each of the equivalent five catalytic cysteines present in the R1 subunit of class I enzymes (Booker & Stubbe, 1993); this has been confirmed by site-directed mutagenesis studies (Booker

et al., 1994). More recently, class II RNR genes have been identified in the genomes of two ancient eubacteria [the radioresistant species *Deinococcus radiodurans* (White *et al.*, 1999) and the deeply rooted hyperthermophilic species *Thermotoga maritima* (Jordan *et al.*, 1997)], in the archaea [*Archaeoglobus fulgidus* (Klenk *et al.*, 1997), *Methanobacterium thermoautotrophicum* (Smith *et al.*, 1997), *Thermoplasma acidophilum* (Tauer & Benner, 1997; Ruepp *et al.*, 2000), *Pyrococcus furiosus* (Riera *et al.*, 1997), *Pyrococcus horikoshii* OT3 (Kawarabayasi *et al.*, 1998), *Aeropyrum pernix* (Kawarabayasi *et al.*, 1999) and *Halobacterium* sp. (Ng *et al.*, 2000)], in the genome databases of several eubacteria [including *Pseudomonas aeruginosa* (Jordan *et al.*, 1999; Stover *et al.*, 2000)], in *Mycobacterium tuberculosis* [where it is referred to as *nrdZ* (Cole *et al.*, 1998)], and in two mycobacterial phages [L5 (Hatfull & Sarkis, 1993) and D29 (Ford *et al.*, 1998)].

Most class II RNRs exhibit significant sequence relatedness among themselves and, to a much lesser extent, with the R1 subunit of class I RNRs. They share with the class I enzymes conservation of the functional cysteines, as well as some features of the allosteric regulation of substrate specificity (Eliasson *et al.*, 1999). These observations have led to the view that class I and II RNRs are likely to possess related tertiary structures and that the genes that encode them may have a common ancestor, despite the marked differences in their overall primary sequences (Reichard, 1993). Possibly, this accounts for the finding of an increasing number of bacterial species that possess more than one RNR, suggesting particular functions for these enzymes in different growth conditions (Jordan & Reichard, 1998; Jordan *et al.*, 1999; Torrents *et al.*, 2000). Thus, a number of aerobic bacteria possess genes encoding both class I and class II RNRs. For example, *Deinococcus radiodurans* contains class Ib and class II RNR genes, although expression of the class Ib genes was not detected (Jordan *et al.*, 1997). Similarly, some *Pseudomonas* spp. contain and express class Ia and class II RNR genes, and also possess genes encoding an anaerobic class III RNR (Jordan *et al.*, 1999). In this context, it is noteworthy that *Mycobacterium tuberculosis*, a member of the same high-G + C branch of the actinomycetes as the genus *Streptomyces*, contains a class Ib RNR gene cluster that is expressed, and a class II RNR gene that, to date, has not been reported to be expressed (Yang *et al.*, 1994, 1997). These and other related findings have generated considerable interest in the further characterization of bacterial RNR genes and enzymes.

In this paper we report the presence of class Ia and class II RNR genes in *Streptomyces* spp. The *Streptomyces clavuligerus nrdJ* gene encodes a 5'-deoxyadenosylcobalamine-dependent class II RNR, and the *nrdAB* genes encode a class Ia RNR. Quantitative measurement of the copy number of the *S. clavuligerus nrdAB* and *nrdJ* mRNAs by real-time PCR shows that the class Ia and class II RNR genes are differentially expressed during vegetative growth.

METHODS

Bacterial strains and plasmids. Strains with NRRL numbers were obtained from the Northern Regional Research Center, Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, IL, USA. *Streptomyces* strains *S. coelicolor* M145 (Hopwood *et al.*, 1985), *S. clavuligerus* DSM 738 (NRRL 3585), *S. lipmanii* (NRRL 3584) and *S. jumonjinensis* (NRRL 5741) were cultured at 30 °C in tryptone soy broth (TSB; Biolife) supplemented with 1% (v/v) glycerol, as described previously (Aharonowitz *et al.*, 1993). *Escherichia coli* XL-1 Blue was a gift from H. Schrempf (University of Osnabrück, Germany); plasmids pBR322 and pUC18 were obtained from MBI Fermentas.

Chemicals and enzymes. Oligodeoxynucleotide primers were obtained from Biotechnology General. Restriction endonucleases, T4 ligase and alkaline phosphatase were purchased from Boehringer Mannheim. *Taq* polymerase was obtained from MBI Fermentas. 5'-Deoxyadenosylcobalamine and other biochemical reagents were purchased from Sigma.

Purification of RNR. Bacteria were grown in TSB medium supplemented with 1% glycerol, at 30 °C in a shaking incubator (250 r.p.m.) to OD₆₀₀ 3–5, harvested by centrifugation and stored at –70 °C. All subsequent manipulations during the purification procedure were carried out at 4 °C. About 30 g (wet weight) of the bacterial cell pellet was thawed and resuspended in 120 ml of a solution containing 50 mM Tris/HCl pH 7.5, 1 mM EDTA, 2 mM DTT, 1 mM PMSF and 5 mg DNase I (Sigma). The suspension was sonicated (3 cycles of 15 s at 4 °C) (Sonicator XL, Misonix), and then centrifuged for 15 min at 15 000 r.p.m. in a Sorvall SS34 rotor. Solid ammonium sulphate was added to the clear supernatant solution to 45% saturation; the mixture was then stirred for 20 min and centrifuged for 15 min as before. The resulting precipitate was dissolved in buffer A (50 mM Tris/HCl pH 8.0, 1 mM EDTA, 2 mM DTT) and an aliquot was kept for the determination of protein concentration and enzyme activity after deionization on a G-25 Sephadex column (Pharmacia). The remaining sample was applied onto a 230 ml Sepharose CL-6B column equilibrated with buffer A, and the column was eluted at a rate of 0.5 ml min⁻¹ with this buffer. Fractions (3 ml) were collected and analysed for protein concentration and enzyme activity. Pooled fractions were further purified by affinity chromatography on a Sepharose-dATP column, prepared by coupling the 2'-deoxyadenosine 5'-(γ -4-aminophenyl) triphosphate sodium salt (USB) to a cyanogen-bromide-activated Sepharose 4B column (Pharmacia) according to the supplier's instructions. The sample was adjusted to 1 mM PMSF, 5 mM DTT and 10 mM CaCl₂ and adsorbed onto an 8 ml Sepharose-dATP column. The column was washed with buffer B (30 mM Tris/HCl, pH 7.5, 1 mM EDTA, 5 mM DTT, 10 mM CaCl₂), followed by the same buffer containing 1 mM ATP. RNR protein was eluted from the column with buffer B containing 1 mM dATP in place of ATP.

RNR activity assay. The standard reaction mixture contained 0.1–0.2 mg protein from the deionized ammonium sulphate precipitation step (referred to as the crude cell extract), or from the Sepharose chromatography step, dissolved in a solution containing 0.56 mM CDP or CTP, 20 μ M 5'-deoxyadenosylcobalamine, 60 μ M dATP, 50 mM DTT, 10 mM CaCl₂ and 50 mM Tris/HCl (pH 8.0) to a final volume of 100 μ l. Incubation was at 30 °C for 25 min and the amount of dCDP or dCTP formed was determined by sequential boronate and anion-exchange HPLC, essentially as described by Hendricks & Mathews (1998).

Peptide sequencing. The dATP eluate from the last purification step was concentrated to a volume of 50 μ l in a Filtron spin-tube (Microsep) and digested with LysC protease (Wako Chemicals) at 37 °C overnight in 0.1 M ammonium bicarbonate (pH 8.0) containing 1 M guanidine hydrochloride, at an endoprotease to protein ratio of 1:10. The cleavage products were separated by reverse-phase liquid chromatography on a Sephasil C8 SC2 2.1/10 column, operated in the SMART system (Pharmacia) using a gradient of 5–60% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.1 ml min⁻¹ over a total time of 90 min. The eluted peptides were subjected to automated Edman degradation in a Procise apparatus (model 491; Applied Biosystems). The sequences and positions of the peptides are shown in Fig. 2(a).

Isolation of the *S. clavuligerus nrdJ* gene. *S. clavuligerus* genomic DNA was extracted as described by Hopwood *et al.* (1985), and a segment of the *nrdJ* gene was amplified by PCR with primers designed according to the internal peptide sequences. Genomic DNA (0.1 μ g) was incubated in a total volume of 50 μ l, together with 50 pmol each primer, all four dNTPs (0.2 mM), 5 μ l 10 \times PCR buffer (MBI Fermentas) and 2 units *Taq* polymerase. The reaction was run in a PTC-100 machine (MJ Research) using the following program: 4 min at 94 °C followed by 29 cycles of 1 min at 94 °C, 2 min at 51 °C and 2 min at 72 °C, and completed with 10 min at 72 °C. Forward [5'-GG(C/G)AACCAGTC(C/G)TT(C/T)GAC-3'] and reverse [5'-CTT(C/G)GT(C/G)GCGCA(C/G)GA(A/G)AA-3'] primers were designed according to the sequence of two *S. clavuligerus* internal peptides (GNQSF and FSCGTK, see Fig. 2a), taking into account the high G + C content of the *Streptomyces* genome. The ~1.6 kb PCR product was purified using the QIAquick PCR Purification kit (Qiagen). To obtain the full-length coding sequence, *S. clavuligerus* genomic DNA (0.68 μ g) was separately digested with restriction endonucleases *Asp*718 and *Bam*HI, electrophoresed on 1% agarose (type II, Sigma), and blotted onto a Hybond-nylon membrane (Amersham). Southern hybridization (Sambrook *et al.*, 1989) and labelling of the 1.6 kb probe was carried out with digoxigenin (DIG), using the DIG DNA-labelling and detection kit (Boehringer Mannheim) according to the manufacturer's instructions. DNA fragments that hybridized with the probe were eluted from 1.2% low-melting-point agarose (type VII, Sigma), purified by phenol-extraction, precipitated with ethanol and ligated into vectors pBR322 (*Bam*HI fragment) and pUC18 (*Asp*718 fragment). *E. coli*(pBR322) transformants containing *S. clavuligerus* RNR sequences were detected by dot-blot hybridization using the DIG-labelled 1.6 kb probe; pUC18 transformants were screened by PCR, employing primers designed to amplify sequences located within the N-terminal coding portion of the 1.6 kb probe. Subcloning and reconstruction of the entire *nrdJ* gene is described in Results.

Detection of DNA by Southern analysis. Chromosomal DNA was isolated from different *Streptomyces* spp. (Procedure 3, Hopwood *et al.*, 1985), digested, electrophoresed and transferred onto nylon membranes using standard procedures (Sambrook *et al.*, 1989). Southern blots were hybridized at high stringency (50% formamide, 42 °C, followed by washing with 2 \times SSC at room temperature for 5 min, 5 min at 42 °C, then twice with 0.5 \times SSC at 60 °C for 15 min) with *nrdJ* and *nrdA* randomly DIG-labelled probes as described in the previous section. The probes were generated by PCR using *S. clavuligerus* genomic DNA and primers 5'-AACATCGTCA-CCAGTAAGTACTTC-3' (forward) and 5'-ACATCGAGA-ATGACCAT-3' (reverse) for *nrdJ*, and 5'-GAGAC(C/G)GC(G/C)GT(C/G)TGCAACCT-3' (forward) and 5'-CTT(C/

G)AGGGCGTC(G/C)AC(G/C)AGGTA-3' (reverse) for *nrdA*. The probes were designed according to conserved nucleotide and amino acid sequences in alignments of the *S. clavuligerus* and *S. coelicolor nrdJ* and *nrdA* genes and their deduced proteins.

RNA extraction. Total RNA was isolated from 1 g wet weight *S. clavuligerus* cells grown to early-, mid- and late-exponential phases in TSB medium supplemented with 1% glycerol using the modified Kirby procedure (Hopwood *et al.*, 1985). RNA samples (250 µl) were treated with 25 U RNase-free DNase (RQ1, Promega), to remove residual traces of contaminating DNA. RNA concentrations were determined by measurement of A_{260} and analysed by electrophoresis in formaldehyde/agarose gels.

Reverse transcriptase and PCR. Two methods were used to prepare cDNA from total RNA. Method 1 employed the RNA LA PCR Kit (version 1.1; Taqara) according to the manufacturer's instructions, and was used in studies to obtain a qualitative measure of *nrdJ* and *nrdAB* transcription and to analyse co-transcription of the *nrdAB* genes. Method 2 was used for the quantitative measurement of *nrdJ* and *nrdAB* transcription using real-time PCR.

In Method 1, RNA (1 µg) in 20 µl containing 1 mM dNTP mix, 5 mM MgCl₂ and 30 pmol *nrdJ* or *nrdA* reverse (antisense) primer (see below) was denatured for 5 min at 80 °C, after which time avian myeloma virus (AMV) reverse transcriptase XL (5 U) and RNase inhibitor (20 U) were added. The reaction was incubated at 55 °C for 45 min and stopped by heating at 99 °C for 5 min. Eighty microlitres of LA PCR buffer containing 2.5 mM MgCl₂, 4% DMSO, 30 pmol forward primer and 2.5 U LA Taq was added to the mixture. PCR was carried out on the mixture using the following procedure: 94 °C for 3 min, 45 cycles of amplification carried out in a thermal cycler, 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, and completed by 72 °C for 10 min. The 20-mer primers used for amplification were 5'-GCGCCCGGATGACCGGC-GAG-3' (forward) and 5'-GCCATGAGCAGCGCCGCGAG-3' (reverse) for *nrdJ*, and 5'-AGACCGCGGTCTGCAACCT-G-3' (forward) and 5'-TGGCCAGGAGCAGGGAGTTG-3' (reverse) for *nrdA*. DNA fragments of 423 bp and 485 bp were produced in PCR reactions using chromosomal DNA as template and the above *nrdJ* and *nrdA* primers. Control samples, in which reverse transcriptase was omitted in RT reactions and in which genomic DNA was used as template in PCR reactions, were run in parallel with RT-PCR reactions. Co-transcription analysis of *nrdA* and *nrdB* genes was performed with cDNA using the forward and reverse primers 5'-GCAGGGCCTGAAGACCACGTAC-3' and 5'-CAGGT-GTTCTTGATGGCGTCC-3', respectively.

In Method 2, RNA (4 µg) in a 20 µl reaction containing 1 × AMV buffer (Promega), 1 mM dNTP mix, 40 pmol *nrdJ* or *nrdA* reverse (antisense) primers (see above) was denatured at 80 °C for 5 min. Ten units of AMV reverse transcriptase and 20 U RNasin (both from Promega) were added and the mixture was incubated at 50 °C for 45 min; the reaction was stopped by heating at 99 °C for 5 min. Control reactions were carried out without reverse transcriptase. Quantitative PCR was carried out with the LightCycler System (Roche) using the LightCycler-FastStart DNA Master SYBR Green I. Labelling of PCR products was performed with the SYBR Green I dye, which fluoresces when bound to double-stranded DNA. The 20 µl reaction contained the LightCycler-FastStart reaction mix and enzyme, 3.5 mM MgCl₂, 0.5 mM 20-mer forward and reverse primers (see above), 5% DMSO and 2 µl cDNA

sample or standard DNA dilutions. Mixes were dispensed into sealed capillary tubes, preincubated at 95 °C for 10 min followed by 40 cycles of amplification at 95 °C for 15 s (temperature transition 20 °C s⁻¹), 65–60 °C for 10 s (step size 0.5 °C, step delay 1 cycle), 72 °C for 20 s (temperature transition 2 °C s⁻¹). The detection of fluorescent *nrdJ* and *nrdA* DNA products was monitored once every cycle at 87 °C and 90 °C, respectively. At these temperatures, any fluorescence signal arising from primer-dimer complexes (which melt at temperatures several degrees lower) is eliminated. Melting-curve analysis was performed in the range 70–98 °C at intervals of 0.1 °C s⁻¹, to confirm that a single DNA PCR product was made from the cDNA template and that it possessed the same T_m as that of the standard control PCR DNA. The copy numbers of *nrdJ* and *nrdA* DNAs were determined by the LightCycler software program from a curve obtained from a plot of the logarithm of the fluorescence versus cycle number, and from a standard curve obtained with serially diluted samples containing 10³–10⁷ copies of the 423 bp *nrdJ* and 485 bp *nrdA* DNA fragments. Copy numbers were calculated after correcting for the presence of non-specific fluorescence in control samples in which reverse transcriptase was omitted.

Sequence and phylogenetic analyses, database searches and deduced protein analysis. Sequence entry, primary analysis and ORF searches were performed using the CloneManager 4.10 program. Primary sequences of class II RNRs were identified in databases using the PAM120 scoring matrix with BLAST algorithms (BLASTP and TBLASTN), as implemented on the NCBI file server (BLAST@ncbi.nlm.nih.gov) (Altschul *et al.*, 1997), and using the FASTA program (Pearson, 1990). Pairwise alignments were performed with the BESTFIT and GAP programs of the Wisconsin Genetics Computer Group package; multiple sequence alignments were made with the CLUSTAL W program (version 1.84; Higgins *et al.*, 1996). Phylogenetic trees were constructed using the PAUP program (Swofford, 2000) and reproduced using TREEVIEW (Page, 1996).

Other methods. Protein concentration was determined by the method of Bradford (1976), with BSA as the standard. SDS-PAGE (0.1% SDS) was performed as described by Laemmli (1970). Gel-filtration chromatography was carried out on a Sephadex 200 HR 10/30 column (Pharmacia) with MW-GF-1000 molecular mass markers (Sigma). Standard methods were used for restriction enzyme digestion and ligation, and transformation of cells by electroporation (Sambrook *et al.*, 1989), unless otherwise stated. Nucleotide sequences were determined using an automatic DNA sequencer (model 377, Applied Biosystems) and a ABI Prism DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems). All sequences reported in this study were obtained from both strands of the DNA.

RESULTS

S. clavuligerus contains a class II RNR

The starting point in this study of the RNR genes of *Streptomyces* spp. was the demonstration that crude cell extracts of *S. clavuligerus* were able to reduce CDP to dCDP only when 5'-deoxyadenosylcobalamine was included in the standard reaction conditions (Table 1). When the cofactor was omitted no class I RNR activity was detected in the extracts. The strict dependence of activity on the presence of 5'-deoxyadenosylcobalamine provides positive evidence for a class II RNR. Activity

Table 1. Dependence of the *S. clavuligerus* RNR activity on 5'-deoxyadenosylcobalamine, DTT and allosteric effectors

RNR activity was measured as the conversion of CDP to dCDP with the Sepharose enzyme preparation in the standard reaction conditions. The complete reaction mixture is described in Methods. Data are the mean values of three independent determinations.

Reaction	Relative activity (%)	Specific activity (nmol dCDP mg ⁻¹ min ⁻¹)
Complete	100	1.85
Minus coenzyme B ₁₂	<0.5	<0.001
Minus DTT	<0.5	<0.001
Minus dATP	<0.5	<0.001
Minus dATP + ATP (2 mM)	48	0.88
Minus Ca ²⁺	17	0.3
Minus Ca ²⁺ + Mg ²⁺ (10 mM)	83	1.54
Minus enzyme	<0.5	<0.001

was dependent on the inclusion, in the standard reaction, of the artificial reductant DTT and the allosteric effector dATP; in the absence of dATP the reaction was strongly stimulated by ATP. Addition of Mg²⁺ and Ca²⁺ stimulated activity, but these cations were not essential. The specific activity of the purified recombinant enzyme (obtained by expressing the class II RNR gene of *S. clavuligerus* in *E. coli*, see below) was several-fold higher for CDP than CTP; when MgCl₂ replaced CaCl₂ the enzyme exhibited a slight preference for CTP over CDP (data not shown). Preliminary experiments in which purified *S. clavuligerus* thioredoxin and thioredoxin reductase plus NADPH were used in place of DTT to drive the reaction were unsuccessful. It was later found that Ca²⁺ inhibited the NADPH-dependent thioredoxin–thioredoxin reductase activity. When Ca²⁺ was omitted from the standard reaction, thioredoxin in the presence of 1 mM DTT caused a three- to fourfold greater stimulation in the conversion of CDP to dCDP than that caused by DTT alone (method 3, Aharonowitz *et al.*, 1993).

The *S. clavuligerus* RNR is an oligomeric protein

The *S. clavuligerus* RNR was purified to near-homogeneity as described in Methods. The protein fractions obtained after Sepharose size-exclusion chromatography retained their activity for several months after purification. Analysis of a Sepharose protein sample by gel filtration on a Pharmacia FPLC machine showed that the activity emerged in a single peak, in a position corresponding to an apparent molecular mass of ~ 430 kDa; the same size was obtained with a sample of the recombinant enzyme (data not shown). Fig. 1 shows the SDS-PAGE images of protein samples taken at each

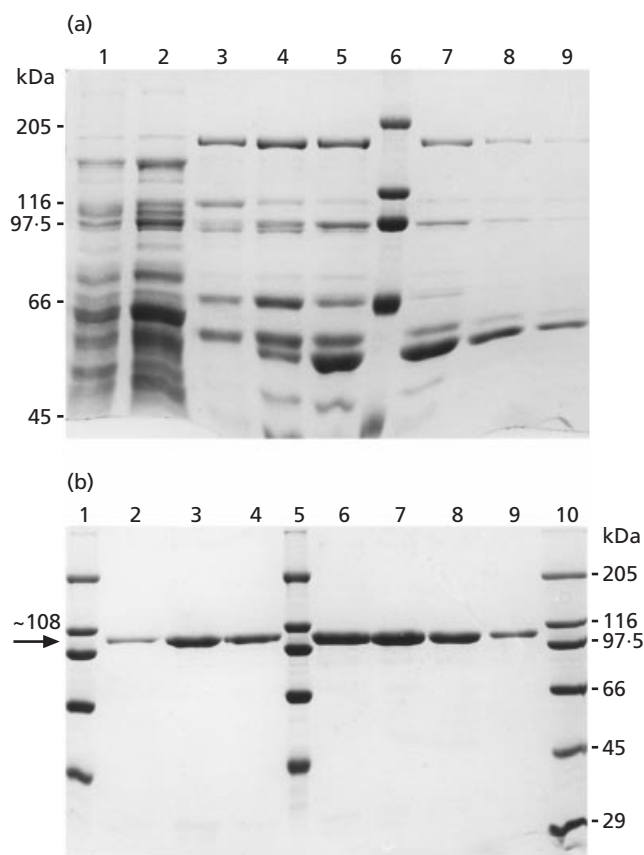


Fig. 1. SDS-PAGE of RNR from *S. clavuligerus* at each step of the purification procedure. (a) Lanes: 1, ammonium sulphate step; 2, Sepharose chromatography step; 3–5 and 7–9, ATP elution step; 6, molecular mass markers. (b) Lanes: 1, 5 and 10, molecular mass markers; 2–4 and 6–9, dATP elution step.

step of the purification procedure. The protein fraction obtained at the final dATP affinity chromatography step had a molecular mass ~ 108 kDa, indicating that the native enzyme in solution probably exists as an oligomer of four identical subunits. The purity of the Sepharose-dATP enzyme preparation was judged to be greater than 95% by SDS-PAGE, and was considered suitable for obtaining amino acid sequence data.

Isolation of the *S. clavuligerus* *nrdJ* gene

Nineteen internal peptide sequences were obtained as described in Methods. A search of the protein databases using the largest of the peptides, ELXSXGXNASGPVS-FMRGADASAG (see Fig. 2a), revealed it to be similar to an ORF encoded in the *Mycobacterium tuberculosis* genome (Cole *et al.*, 1998) that had been assigned as a protein of unknown function but was similar to the RNR large subunit (AL021942). Thirteen of the 21 peptide residues (three unidentified) matched those in the *M. tuberculosis* sequence. When the corresponding peptide sequence in the *M. tuberculosis* ORF was used to search the databases for related protein sequences, the

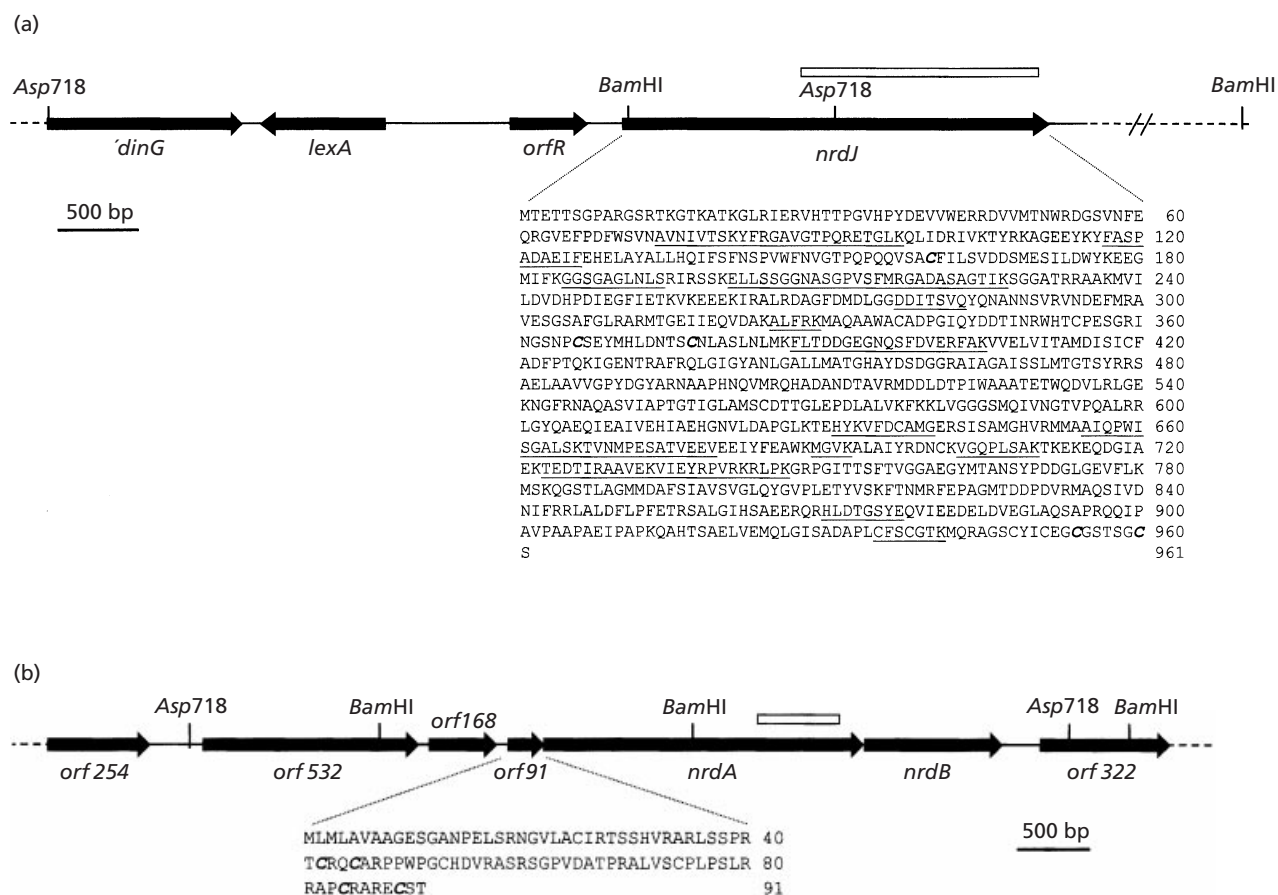


Fig. 2. (a) Schematic organization of genes in the *S. clavuligerus* chromosome, containing the class II RNR *nrdJ* gene and showing the deduced amino acid sequence. The *Bam*HI restriction fragment contains all but the first 10 codons of the 2883 nt that compose the *nrdJ* structural gene; the *Asp*718 restriction fragment overlaps with the N-terminal coding portion of *nrdJ* and includes its putative promoter upstream region, *orfR*, *lexA* and part of *dinG*. Directionality of the individual ORFs is shown by thick arrows. An open rectangular box indicates the probe used in Southern blot analysis. The deduced amino acid sequence of the *nrdJ* gene is shown with the positions of the peptides that were sequenced underlined; the five catalytic cysteines are indicated in bold italics. (b) Schematic organization of genes in the *S. coelicolor* M145 chromosome, containing the class Ia RNR *nrdAB* genes. The *Asp*718 fragment contains the *nrdA* and *nrdB* genes, which overlap and code for the large and small subunits of class Ia RNR, respectively. *orf91* overlaps with *nrdA* and encodes a hypothetical 91 aa protein containing two pairs of cysteines (shown in bold italics in the deduced amino acid sequence), which are present in sequences resembling those of redox-active proteins. *orf168* encodes a putative acetyltransferase from the GNAT family. *orf532* and *orf254* are located upstream of *orf168*, the former encodes a putative transporter protein similar to the *E. coli* proline permease PutP, and the latter encodes a putative transcriptional regulator protein from the GntR family. Downstream of *nrdB* is located *orf322*, which encodes a putative transcriptional regulator of the AraC family. Arrows show the directionality of the individual ORFs. An open rectangular box indicates the probe used in Southern blot analysis.

highest similarity (82%) was found with an unannotated *S. coelicolor* ORF (Sanger Centre, cosmid 4H2). Less similarity was observed with other ORFs reported as encoding class II RNRs (see below). The sequences of the *S. clavuligerus* peptides were in excellent accord with the nucleotide sequence deduced from the cloned gene (described below). The sequence of the cloned gene was very similar to that encoded in the unannotated 967 aa ORF generated by the *S. coelicolor* genome sequencing project, which was deposited while this work was in progress. The peptide sequence data support the view that the *S. clavuligerus* coenzyme-B₁₂-dependent RNR is a class II enzyme.

A ~1.6 kb DNA fragment of the *S. clavuligerus nrdJ* gene was obtained by PCR, as described in Methods. Nucleotide sequencing showed it to contain an un-interrupted ORF with a deduced translation product closely resembling that present in an unannotated *S. coelicolor* ORF (cosmid 4H2). To clone the entire *nrdJ* gene, and the flanking sequences necessary for its expression, the 1.6 kb fragment was labelled with digoxigenin and used as a probe for Southern analysis of *S. clavuligerus* genomic DNA. Separate digests with *Bam*HI and *Asp*718 produced in each case a single hybridization band corresponding to fragments of ~12 kb and ~6 kb, respectively (data not shown). The

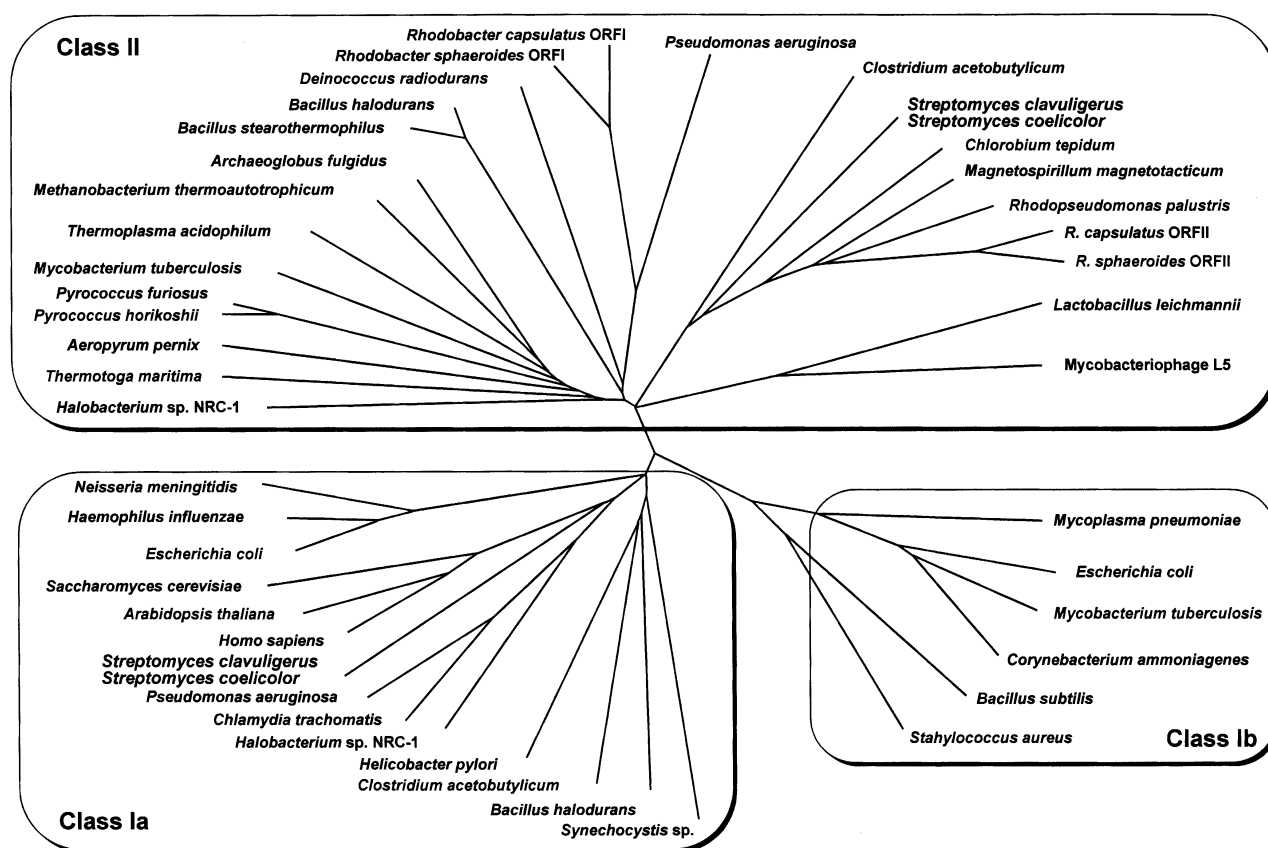


Fig. 3. Phylogenetic analysis of deduced amino acid sequences of known and putative class I and class II RNRs. Names of organisms, accession numbers of annotated ORFs and sources for unannotated ORFs are as follows: class II RNRs, *Aeropyrum pernix* K1 (AP000063), *Archaeoglobus fulgidus* (AE000988), *Bacillus halodurans* (AP001516), *Bacillus stearothermophilus* (data from <http://www.genome.ou.edu/bstearo.html>), *Chlorobium tepidum* (data from <http://www.tigr.com/>), *Clostridium acetobutylicum* (data from <http://www.cric.com/>), *Deinococcus radiodurans* R1 (AE002068), *Halobacterium* sp. NRC-1 (AE005073), *Lactobacillus leichmannii* (L20047), *Magnetospirillum magnetotacticum*, *Rhodobacter capsulatus*, *Rhodobacter sphaeroides*, *Rhodopseudomonas palustris* (preliminary sequence data for these bacteria were obtained from The DOE Joint Genome Institute at http://spider.jgipf.org/JGI_microbial/html/), *Methanobacterium thermoautotrophicum* (AE000845), *Mycobacterium tuberculosis* H37Rv (AL021942), mycobacterial phage L5 (Z18946), *Pseudomonas aeruginosa* PAO1 (AE004962), *Pyrococcus furiosus* (U78098), *Pyrococcus horikoshii* (AP000002), *Streptomyces clavuligerus* (AJ224870), *Streptomyces coelicolor* A3(2) (AL022268), *Thermoplasma acidophilum* (AL445067), and *Thermotoga maritima* (AE001698); class Ia RNR large subunits, *Arabidopsis thaliana* (AF092841), *Bacillus halodurans* (AP001508), *Chlamydia trachomatis* (AE001355), *Clostridium acetobutylicum* (data from <http://www.cric.com/>), *Escherichia coli* (X06999), *Haemophilus influenzae* (U32839), *Halobacterium* sp. NRC-1 (AE005120), *Helicobacter pylori* J99 (AE001494), *Homo sapiens* (AF107045), *Neisseria meningitidis* (AL162756), *Pseudomonas aeruginosa* PAO1 (AE004545), *Saccharomyces cerevisiae* (U18813), *Streptomyces clavuligerus* (AJ277778), *Streptomyces coelicolor* A3(2) (AJ276618 and AL359214), and *Synechocystis* sp. PCC6803 (D90913); class Ib RNR large subunits, *Bacillus subtilis* (Z68500), *Corynebacterium ammoniagenes* (Y09572), *Escherichia coli* (AE000352), *Mycobacterium tuberculosis* H37Rv (AL021287), *Mycoplasma pneumoniae* (AE000050), and *Staphylococcus aureus* BB (AJ133495).

*Bam*HI and *Asp*718 DNA fragments were ligated into vectors pBR322 and pUC18, respectively, and used to transform *E. coli*. Plasmids containing *nrdJ* sequences were identified in mini-libraries by PCR, using primers based on the sequence of the probe. Approximately 4.5 kb of the DNA insert in one of the pBR322 derivatives (pBam42) was sequenced from both ends and found to contain a long ORF of 951 aa with no initiation codon, followed by a termination codon and a potential stem-loop structure. Sequencing of the ends of the ~1.4 kb DNA insert in one of the pUC18 derivatives (pAsp158) revealed it to contain a sequence that

overlapped with, and extended upstream of, the N-terminal portion of the *nrdJ* gene. A physical map of the genomic region containing the *nrdJ* gene and the position of the restriction sites used in its reconstruction are shown in Fig. 2(a).

Molecular phylogenetic analysis of the *Streptomyces* class II RNR

The deduced amino acid sequence of the *S. clavuligerus* *nrdJ* gene (AJ224870) is shown in Fig. 2(a). The structural gene consists of 2883 bp and encodes a

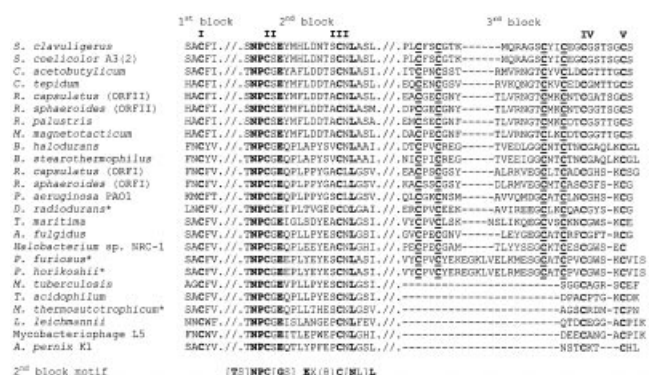


Fig. 4. Abridged alignment of selected class II RNRs to illustrate the positional relationship of conserved cysteines. The deduced amino acid sequences of 25 class II RNR proteins are shown. Organisms, accession numbers and relevant sources of sequences are given in the legend to Fig. 3. Fully conserved amino acid residues are shown in bold. In II and III, indicate the positions of the two active-site cysteines which directly reduce the nucleotide substrate; II, indicates the position of the cysteine postulated to be oxidized to a thiyl radical; IV and V, indicate the positions of cysteines proposed to be responsible for the transfer of reducing equivalents to the active site. Other cysteines mentioned in the text are shown in bold and underlined. The sequence motif common to class II RNRs is shown at the bottom. Asterisks (*) denote the presence of an intein sequence located between cysteine II and the adjacent glycine.

polypeptide of 961 aa, with a predicted molecular mass of 104.791 kDa and a pI of 5.25. The *nrdJ* has a G + C content of 66.7 mol% and has a codon usage that is typical for streptomycete genes (Bibb *et al.*, 1984). A well-conserved ribosome-binding site, GGAGG (Strohl, 1992), is located 6 nt upstream of the start codon and a 27 bp imperfect palindrome is present just downstream of the translation stop codon. Pairwise comparisons show that the deduced amino acid sequence of the *S. clavuligerus nrdJ* gene shares 92% similarity (percentage identical residues) with that predicted for the *S. coelicolor* ORF (AL022268). *S. clavuligerus nrdJ* shares 37% similarity with a 987 aa ORF from *Clostridium acetobutylicum*, annotated as an RNR, 31% similarity with an unannotated 1154 aa ORF of *Chlorobium tepidum*, identified in database searches as encoding a putative class II RNR, and 20–30% similarity with several other eubacterial and archaeal class II RNRs. No statistically significant alignments could be made with the *Lactobacillus leichmannii* RNR or with either of the RNRs encoded by the mycobacterial phages L5 and D29. Fig. 3 depicts the molecular phylogenetic analysis of the deduced amino acid sequences of known class II RNR genes and homologues detected in databases during this work.

A class II RNR sequence motif

The *S. clavuligerus* class II RNR contains 15 cysteines, five of which (Cys160, Cys366, Cys377, Cys954 and Cys960) correspond to catalytic cysteines based on a comparison with the positions of the known catalytic

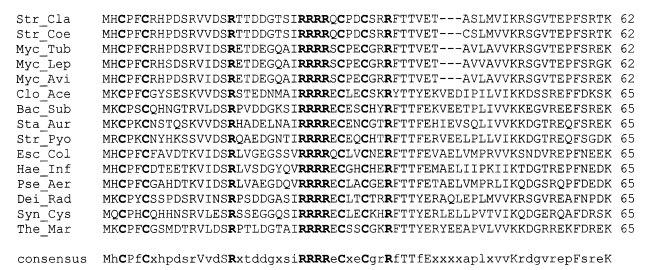


Fig. 5. Alignment of *Streptomyces* spp. OrfR proteins and homologues. The deduced amino acid sequences of the N-terminal portions are shown. Organisms, accession numbers and database references are: *Streptomyces clavuligerus* (AJ224870), *Streptomyces coelicolor* (AL022268), *Mycobacterium tuberculosis* (Z96072), *Mycobacterium leprae* (U00019), *Mycobacterium avium* (data from <http://www.tigr.com/>), *Clostridium acetobutylicum* (data from <http://www.cric.com/>), *Bacillus subtilis* (AF008220), *Staphylococcus aureus* and *Streptococcus pyogenes* (data for both from <http://www.genome.ou.edu/>), *Escherichia coli* (X64395), *Haemophilus influenzae* (U32775), *Pseudomonas aeruginosa* (AE004822), *Deinococcus radiodurans* (AE001872), *Synechocystis* sp. PCC6803 (D64000), and *Thermotoga maritima* (AE001810). The 'consensus' shows complete conservation (upper-case letters), incomplete conservation (lower-case letters), any amino acid (x), conserved arginines (R) and cysteines (C) among the proteins studied.

cysteines in the *Escherichia coli* and *Lactobacillus leichmannii* class I and class II RNR sequences. Cys366 is presumed to be involved in generating the thiyl radical, Cys160 and Cys377 are thought to be involved in substrate reduction, and Cys954 and Cys960 to be involved in the acceptance of electrons from the cellular hydrogen donor system. Fig. 4 shows an abridged alignment of the known primary amino acid sequences of class II RNRs to illustrate the positional relationship of the catalytic cysteines and some other highly conserved cysteines; it includes the deduced sequences of peptide segments of several ORFs predicted to encode class II RNRs that were recovered from nucleotide databases while this work was in progress. Class II RNRs all possess a fixed bridge of 10 aa separating the second and third catalytic cysteines. In class I RNRs this number is variable, containing 13–14 residues in most eukaryotic and viral class I RNRs, and 14–37 and 25–26 residues in most prokaryotic class Ia and Ib RNRs, respectively (Kreisberg-Zakarin, 1999). The consensus peptide sequence (TS)NPC(GS)EX₆C(NL)L (fully conserved residues are indicated in bold) was used to probe the protein databases and uniquely identifies class II RNRs. Another feature indicative of *Streptomyces* class II RNRs, and the putative RNRs from *Bacillus halodurans*, *Bacillus stearothermophilus*, *Clostridium acetobutylicum*, *Chlorobium tepidum*, *Rhodobacter capsulatus*, *Rhodobacter sphaeroides*, *Rhodospseudomonas palustris* and *Magnetospirillum magnetotacticum*, is the presence of a bridge of five residues separating the fourth and fifth catalytic cysteines. In other class II RNRs two or four residues separate these cysteines. Four other closely spaced cysteines are also found in the region just upstream of the C-terminal pair of catalytic cysteines in

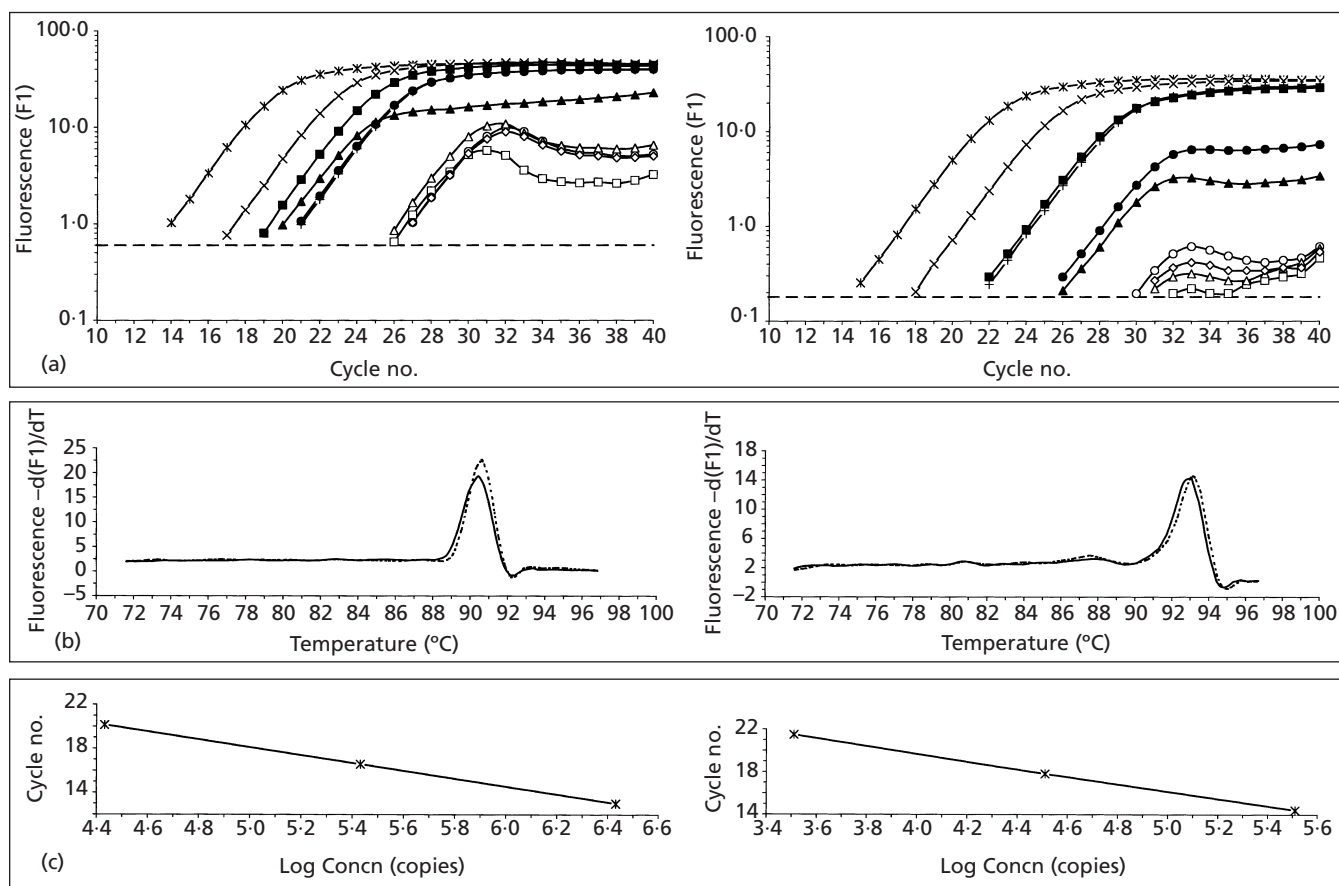


Fig. 6. Quantitative real-time PCR measurement of *S. clavuligerus* class Ia *nrdAB* and class II *nrdJ* cDNAs produced after reverse transcription (RT) of 400 ng total RNA extracted at the early-, mid- and late-exponential phases of growth. Panels (a, b, c) show a representative analysis of the transcription of *nrdJ* and *nrdAB* genes using the LightCycler System. (a) Logarithmic plot of fluorescence (F1) versus cycle number. Data for *nrdJ* and *nrdAB* are represented by the left and right graph, respectively. Symbols: \times , \times , +, copy number of DNA standards; \blacksquare and \square , RNA samples isolated at the early-exponential phase of growth with and without RT, respectively; \bullet and \circ , RNA samples isolated at the mid-exponential phase of growth with and without RT, respectively; \blacktriangle and \triangle , RNA samples isolated at the late-exponential phase of growth with and without RT, respectively; \diamond , control sample without template. Copy numbers of DNA standards for *nrdJ* are 2.70×10^6 (\times), 2.70×10^5 (\times) and 2.70×10^4 (+), and 3.24×10^5 (\times), 3.24×10^4 (\times) and 3.24×10^3 (+) for *nrdAB*. The broken line indicates the baseline. (b) Melting-curve analysis. The solid line indicates results when *nrdJ* (left graph) and *nrdAB* (right graph) were present at a copy number of 2.7×10^4 and 3.24×10^3 , respectively. The dotted line indicates results for *nrdJ* and *nrdAB* during the early-exponential phase of growth. (c) Crossing points (cycle number) of log linear correlations with the baseline, plotted against the logarithmic concentration of the standards for *nrdJ* (left) and *nrdAB* (right).

S. clavuligerus (Cys935, Cys938 and Cys948, Cys951), in *S. coelicolor* and in most other, but not all, class II RNRs. However, the function of these cysteines is unknown.

orfR located upstream of *nrdJ* encodes a putative redox-like protein

Immediately upstream of *nrdJ*, in the same orientation, is an ORF encoding a protein of 172 aa and of unknown function. Inspection of the protein databases showed that this putative protein is present throughout the eubacteria, but is not present in archaea. A multiple amino acid sequence alignment revealed the protein encoded by the ORF to possess several noticeable features, Fig. 5. First, it contains two pairs of cysteines

separated by a bridge of two amino acids in a sequence that is reminiscent of the redox-active site present in thioredoxins and glutaredoxins. Second, it contains a run of four consecutive arginine residues adjacent to one of the cysteine pairs. Sequence similarity among different bacterial ORFs is mainly confined to the N-terminal part of the molecule. Because of the presence of the two cysteine pairs, we assume that the putative protein may have a redox activity and have designated it OrfR to indicate its possible redox-regulatory role and for the unusual arginine (R) run it contains. Upstream of *orfR*, oriented in the opposite direction, is an ORF encoding the LexA global regulator, and beyond it an ORF encoding an ATP-dependent helicase. In *Mycobacterium tuberculosis*, *lexA* and *orfR* map well away from the class-II-like RNR *nrdZ* gene (Cole *et al.*, 1998).

***Streptomyces* spp. contain class Ia RNR genes**

During the course of this work we identified an unannotated sequence (cosmid 7E4) in the *S. coelicolor* nucleotide database, containing two adjacent ORFs. The deduced proteins of these ORFs resembled the protein subunits of class Ia RNRs. Fig. 2(b) shows the organization of the *S. coelicolor* DNA region containing the genes, designated *nrdA* and *nrdB*. Southern analysis and DNA sequencing showed that *S. clavuligerus* (AJ277778), *S. lipmannii* (AJ295339) and *S. jumonjensis* (AJ295338) possess homologous *nrdAB* genes that exist in single copy (data not shown). Immediately upstream of *nrdA*, and overlapping with it, is an ORF encoding a putative 91 aa protein of unknown function, containing two pairs of cysteines resembling those present in redox-active proteins. In *S. coelicolor* the *nrdA* TAA termination codon overlaps with the *nrdB* ATG start codon by one nucleotide; in *S. clavuligerus* four nucleotides, ATGA, contain the *nrdA* TGA termination codon and the *nrdB* ATG start codon. The *S. coelicolor nrdA* and *nrdB* genes were cloned and expressed in *E. coli*. Although we were unable to detect class I activity in crude cell extracts (see above), NrdA and NrdB recombinant proteins were able to convert CDP to dCDP; moreover the activity was completely abolished by treatment with 5 mM hydroxyurea, which is diagnostic for class I RNRs (data not shown).

Comparison of the deduced amino acid sequences of the *Streptomyces* NrdA and NrdB proteins showed them to be more similar to the subunits of the large R1 and small R2 proteins of RNRs of plants and mammals (*P*-values determined with the BLAST program are $\sim e^{-155}$ and e^{-54} , respectively) than to eubacterial class Ia RNR subunits (*P*-values of $\sim e^{-49}$ and e^{-13} for *E. coli* NrdA and NrdB, respectively), and even less similar to the subunits of eubacterial class Ib enzymes (NrdE and NrdF). Fig. 3 depicts the molecular phylogenetic analysis of the large subunit of class I RNRs and shows that the *Streptomyces* NrdA protein is more closely related to the R1 protein of plants and animals than to the typical NrdA and NrdE proteins of eubacteria.

Differential transcription of the class Ia and class II RNR genes of *S. clavuligerus*

To determine the expression of class Ia and II RNR genes during growth in liquid culture (representing vegetative growth) total RNA was isolated from cultures of *S. clavuligerus* grown in TSB medium to early-, mid- and late-exponential phase, and subjected to reverse transcription with antisense primers, and the cDNAs were analysed by PCR using primers specific to the *nrdJ* and *nrdA* genes. PCR products were of the expected size and were sequenced to confirm their origin. Qualitative RT-PCR was first used to demonstrate co-transcription of the *nrdA* and *nrdB* genes. Primers designed according to *nrdA* (nucleotides 2247–2268, GenBank AJ277778) and *nrdB* (nucleotides 2530–2510, GenBank AJ277778) were used to amplify cDNA obtained after subjecting total RNA to reverse transcriptase. The amplified DNA

Table 2. Copy numbers of *S. clavuligerus nrdJ* and *nrdAB* transcripts in vegetative growth

Copy numbers of cDNA per 400 ng total RNA were obtained after reverse transcription. The copy numbers are the mean values of two measurements of each of two independent sets of RNA preparations with a standard mean variation of ± 15 –25%.

Growth phase	Copy number	
	<i>nrdJ</i>	<i>nrdAB</i>
Early-exponential	5.0×10^5	2.8×10^4
Mid-exponential	3.5×10^5	4.0×10^3
Late-exponential	5.0×10^5	1.1×10^3

fragment was estimated by gel electrophoresis to be ~ 280 nt, in agreement with the expected value of 283 nt. Sequencing verified that the cDNA was derived by co-transcription of the overlapping *nrdA* and *nrdB* genes. Qualitative RT-PCR analysis indicated that *nrdJ* mRNA was present throughout the exponential phase of growth, whereas *nrdAB* mRNA was detected at the early-exponential phase of growth but was barely detectable at the mid- and late-exponential phases of growth (data not shown).

Quantitative measurement of *nrdJ* and *nrdA* mRNA levels in exponentially growing cultures of *S. clavuligerus* was performed employing real-time PCR. Aliquots of total RNA extracted from exponentially growing cells were treated with reverse transcriptase; the cDNAs were amplified using the LightCycler System. Fig. 6(a) shows a representative analysis for the detection by fluorescence of *nrdJ* and *nrdA* amplicons from cDNAs made from total RNA prepared at the early-, mid- and late-exponential phases of growth; Fig. 6(b) shows melting-curve analysis of the amplicons; Fig. 6(c) shows a standard curve for estimating copy numbers (see Methods). The presence of contaminating chromosomal DNA in RNA samples was assessed in control PCR reactions in which the reverse transcriptase step was omitted.

Table 2 shows the calculated copy numbers of *nrdJ* and *nrdAB* cDNAs obtained after reverse transcription of RNA extracted at the early-, mid- and late-exponential phases of growth. Copy numbers are the mean values for two measurements of each of two independent sets of RNA preparations, with a standard mean variation of ± 15 –25%. The *nrdJ* copy number was approximately constant throughout the course of exponential growth. In contrast, the *nrdA* copy number, at the same growth points, fell markedly with the progression of the culture towards the mid- and late-exponential phases of growth. Even at the early-exponential phase of growth the number of *nrdA* copies was 10- to 20-fold lower than that of *nrdJ*. Fluorescence levels in control reactions in which reverse transcriptase was omitted were about the same as those in control standard reactions in which the

nucleic acid template was replaced with water, indicating the absence of any significant contaminating chromosomal DNA. Background levels of fluorescence were about 1% for the detected *nrdJ* cDNA, and about 5% for the detected *nrdA* cDNA at the mid- and late-exponential phases of growth.

DISCUSSION

Streptomyces spp. contain class I and class II RNR genes

The existence in *Streptomyces* spp. of genes encoding two different classes of RNR raises challenging questions about their respective roles in the growth cycle. We first summarize the evidence for the occurrence of RNRs in *Streptomyces* spp. and in related actinomycete genera. A class II RNR was first purified from *Streptomyces aureofaciens* and was found to have a molecular mass of 64 kDa and to function as a dimer (Kollarova *et al.*, 1983; Racay & Kollarova, 1996). The *S. clavuligerus* class II RNR described in this work is larger: it has a molecular mass of ~108 kDa and is probably a tetramer. *Streptomyces griseus*, *Streptomyces olivaceus* and *Streptomyces spheroides* (Pryanishnikova & Jordan, 1998) all appear to possess class II RNRs, based on physiological studies which show that *in vivo* DNA synthesis depends on the presence of coenzyme B₁₂ in the growth medium. Class II RNRs have also been characterized from *Corynebacterium nephridii*, with a molecular mass of ~200 kDa and composed of two subunits (Tsai & Hogenkamp, 1980), and from *Propionibacterium shermanii* (Jordan and Petukhova, 1995). With the exception of *S. clavuligerus*, the genes encoding these RNRs have not been cloned. *Mycobacterium tuberculosis* contains a gene, termed *nrdZ*, which is assigned as encoding a class II RNR homologue; we identified in the genome of *Mycobacterium bovis* an unannotated sequence encoding a class II RNR and in the genome of *Mycobacterium leprae* a sequence which appears to represent an incomplete portion of a class II RNR (both sequences from The Sanger Centre, ftp://ftp.sanger.ac.uk/pub/pathogens/). Genes encoding putative class II RNRs are also found in the mycobacterial phages L5 and D29. Therefore, class II oxygen-independent RNRs are evidently widely distributed among the main subgroups of the high-G+C branch of the actinomycetes.

The existence of class I RNRs in actinomycetes is less well documented. *Mycobacterium tuberculosis* contains *nrdE*- and *nrdF*-like genes (Yang *et al.*, 1997) that encode a functional RNR which is related to the class Ib RNR encoded by the *E. coli*, *Salmonella typhimurium* and *Lactococcus lactis nrdEF* genes. Other *Mycobacterium* spp., *M. avium*, *M. leprae* and *M. bovis*, contain in their genomes *nrdEF*-like genes, encoding putative class Ib RNRs. A class Ib manganese-dependent RNR occurs in *Corynebacterium ammoniagenes* (Fieschi *et al.*, 1998). In this work we show that *Streptomyces* species contain genes encoding a class Ia RNR. The genes, denoted as *nrdA* and *nrdB*, were initially identified in an unannotat-

ed sequence in the *S. coelicolor* genome and subsequently isolated from *S. clavuligerus*; they were also shown to be present in other streptomycetes. The *nrdAB* and *nrdJ* genes occur in the *AseI*-b fragment of the standard *S. coelicolor* (A3)2 genome map. In *Mycobacterium* spp. the *nrdE* and *nrdF* genes are not physically linked, whereas in *Streptomyces* spp. the *nrdAB* genes are linked in a similar arrangement to that of the *nrdEF* genes in *E. coli*, *S. typhimurium* and *L. lactis*, and are coordinately expressed from a single transcription promoter. Some other differences between the actinomycetes class I genes and proteins are mentioned below.

While all class I RNRs obtain their reducing power from both thioredoxin and glutaredoxin, few reports exist on their ability to function with class II RNRs. *Streptomyces* spp. lack glutathione (Aharonowitz *et al.*, 1993) and we expected that they would employ thioredoxin, together with thioredoxin reductase and NADPH, as the hydrogen donor system. Thioredoxin was shown to be the hydrogen donor for the *L. leichmannii* and the cyanobacterium *Anabaena* B₁₂-dependent class II RNRs (Booker & Stubbe, 1993; Gleason & Holmgren, 1981). However, thioredoxins C-1 and C-2, isolated from the actinomycete *Corynebacterium nephridii*, were unable to support substrate reduction for their homologous class II RNR (McFarlan *et al.*, 1989). Initially, we observed that the *S. clavuligerus* thioredoxin system was unable to reduce the homologous class II RNR. Subsequently, we found that the presence of divalent metal ions in the standard assay inhibited the activity of the thioredoxin system, and when they were removed thioredoxin was able to weakly stimulate the activity of the class II RNR. Mycothiol, which occurs in millimolar concentrations in many actinomycetes (Newton *et al.*, 1996), may function as a hydrogen donor for the class II RNR, whereas thioredoxin, according to recent studies, may function primarily in streptomycetes and mycobacteria to control the intracellular thiol-disulfide redox status (Paget *et al.*, 1998). Another possible candidate as a hydrogen donor for the class II RNR, OrfR, is suggested by the fact that it possesses two pairs of vicinal cysteines, organized like those in glutaredoxin and thioredoxin. The positional relation of the eubacterial *orfR* to *nrdJ* resembles that of *nrdH* to *nrdEF* in class Ib RNR gene clusters, where the product of *nrdH* is a glutaredoxin-like protein with specificity for class Ib RNRs (Jordan *et al.*, 1996; Jordan *et al.*, 1997). Curiously, the extremely thermophilic Gram-positive *Carboxydotherrmus hydrogeniformans* has an *orfR* homologue that is located immediately upstream of, and overlaps with, the *nrdD* gene of the class III RNR gene cluster (TIGR database). However, NrdH and OrfR show little sequence relatedness. OrfR contains an unusual sequence consisting of four consecutive arginines that are totally conserved in the deduced amino acid sequences in other members of this family (Fig. 5). The role of these arginines is unknown; the fact that the N-terminal portion of the molecule is extremely hydrophilic and contains many charged residues suggests that it may act as a regulatory binding protein.

Class Ia and class II RNR genes of *S. clavuligerus* are differentially transcribed in vegetative growth

Streptomyces and *Mycobacterium* genomes possess genes encoding class I and class II RNRs (www.sanger.ac.uk/Projects/S_coelicolor; Cole *et al.*, 1998). The *M. tuberculosis nrdEF* genes form a biologically active class Ib RNR (Yang *et al.*, 1994, 1997), but it is not known whether the *M. tuberculosis nrdZ* gene encodes a functional class II RNR. Northern analysis was initially used to monitor *S. clavuligerus nrdJ* and *nrdA* mRNA in vegetative growth, but transcripts were not detected in either case. When total RNA was prepared at the early-, mid- and late-exponential phases of growth and analysed by RT-PCR, DNA products were readily detected with *nrdJ* primers, whereas *nrdA* primers revealed DNA products only in the RNA sample prepared at the early-exponential phase of growth.

Real-time PCR permitted a quantitative measure of the copy number of *nrdAB* and *nrdJ* transcripts, and revealed that the number of copies of *nrdJ* mRNA was approximately constant over the entire course of exponential growth, whereas the copy number of *nrdAB* mRNA was at least tenfold less than that of *nrdJ* in the early stages of growth and dropped markedly in later stages. Presumably, the low level of *nrdAB* transcripts accounts in part for our inability to detect class I RNR activity in cell extracts.

Based on these results, we propose that streptomycetes employ two RNRs: a class Ia oxygen-dependent RNR and a class II oxygen-independent RNR that function at different stages in the growth cycle. For example, the class Ia RNR might operate primarily in the early stages of growth following spore germination, whereas the class II RNR might act primarily during vegetative growth. Vegetative growth of *Streptomyces* spp. occurs mainly by cell-wall extension at hyphal tips, with lateral branching. As the culture grows numerous changes take place, resulting in the formation of a dense mycelial pellet, probably in response to nutrient limitation or other physiological stresses including oxygen depletion. The younger and older parts of the mycelium are not physically homogeneous and may be subject to different degrees of oxygen availability. Thus, the existence of two classes of RNRs that differ in their dependence on oxygen may be necessary for proper growth and development. An analysis of the transcription pattern of *nrdJ* and *nrdAB* genes over a wide variety of physiological conditions and the effects on growth and development of gene inactivation should help clarify this matter.

Molecular phylogenetic analysis of RNRs from actinomycetes reveals unexpected divisions

Some unusual features of the RNRs of *Streptomyces* spp. are evident from phylogenetic analysis of the deduced amino acid sequences of the class Ia and class II RNR genes (Fig. 3). The *S. clavuligerus* and *S. coelicolor* class II RNRs belong to a group that, to date, contains

putative class II RNRs that we identified in unannotated sequences in nucleotide databases from *Clostridium acetobutylicum*, *Chlorobium tepidum*, *Rhodobacter capsulatus*, *Rhodobacter sphaeroides*, *Rhodospseudomonas palustris* and *Magnetospirillum magnetotacticum*; significantly, it does not include the putative *Mycobacterium tuberculosis* class II RNR. In fact, the *Mycobacterium tuberculosis* class II RNR is part of a separate and distinct group that exclusively contains archaeal RNRs, while a third group contains the *Lactobacillus leichmannii* RNR and two mycobacterial phage RNRs. The origin of these divisions is obscure. A feature of the group containing the *Streptomyces* spp. class II RNRs is the presence of a characteristic spacing of five residues separating two catalytic cysteines in the C-terminus of the RNRs, other class II RNRs have corresponding spacings of two or four residues. Interestingly, the *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* genomes contain two putative class II RNRs. One ORF contains a four residue spacing, whereas the second ORF contains a five residue spacing; phylogenetic analysis places the former with the archaeal RNRs and the latter with the *Streptomyces* spp. RNRs (Fig. 3). Another surprise is the finding that the predicted *Streptomyces* class Ia RNR, but not other actinomycetes class I RNRs, such as those from *Mycobacterium tuberculosis* and *Corynebacterium ammoniagenes*, is phylogenetically more closely related to its eukaryotic counterpart than to the eubacterial class Ia and class Ib RNRs (other examples noted were *Pseudomonas aeruginosa* and *Chlamydia trachomatis* whose genomes include ORFs resembling the eukaryotic class I RNR). This finding probably explains our failure to detect the *Streptomyces* spp. *nrdAB* genes using probes based on the bacterial class Ia and class Ib RNR genes. What could account for these divisions? Gene transfer events might be responsible for the similarity of the *Streptomyces* spp. *nrdAB* and eukaryotic class I RNRs. In this respect it is curious that a halophilic archaeal genome, which like *Streptomyces* is high in G + C (Ng *et al.*, 2000), contains an ORF encoding a putative class Ia RNR (and an *orf* encoding a class II RNR) that is similar to the class Ia RNR present in *Streptomyces* spp. and eukaryotes, suggesting a possible gene transfer event. In fact, a horizontal gene transfer of the catalase-peroxidase gene has been proposed to have occurred between archaea and eubacteria, including *Streptomyces* spp. and *Mycobacterium* spp. (Faguy & Doolittle, 2000). A more exhaustive analysis of phylogenetic reconstructions of other subgroups of RNR genes, and their proteins, from different actinomycetes, and biochemical characterization of the properties of the *Streptomyces* spp. class Ia RNR, will be needed before this and related questions can be adequately addressed.

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