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NrdR Controls Differential Expression of the *Escherichia coli* Ribonucleotide Reductase Genes▽†

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***Escherichia coli* possesses class Ia, class Ib, and class III ribonucleotide reductases (RNR). Under standard laboratory conditions, the aerobic class Ia *nrdAB* RNR genes are well expressed, whereas the aerobic class Ib *nrdEF* RNR genes are poorly expressed. The class III RNR is normally expressed under microaerophilic and anaerobic conditions. In this paper, we show that the *E. coli* YbaD protein differentially regulates the expression of the three sets of genes. YbaD is a homolog of the *Streptomyces* NrdR protein. It is not essential for growth and has been renamed NrdR. Previously, *Streptomyces* NrdR was shown to transcriptionally regulate RNR genes by binding to specific 16-bp sequence motifs, NrdR boxes, located in the regulatory regions of its RNR operons. All three *E. coli* RNR operons contain two such NrdR box motifs positioned in their regulatory regions. The NrdR boxes are located near to or overlap with the promoter elements. DNA binding experiments showed that NrdR binds to each of the upstream regulatory regions. We constructed deletions in *nrdR* (*ybaD*) and showed that they caused high-level induction of transcription of the class Ib RNR genes but had a much smaller effect on induction of transcription of the class Ia and class III RNR genes. We propose a model for differential regulation of the RNR genes based on binding of NrdR to the regulatory regions. The model assumes that differences in the positions of the NrdR binding sites, and in the sequences of the motifs themselves, determine the extent to which NrdR represses the transcription of each RNR operon.**

Ribonucleotide reductases (RNRs) are essential enzymes in all living cells, providing the only known de novo pathway for the biosynthesis of deoxyribonucleotides, the immediate precursors of DNA synthesis and repair (34). Three major classes of RNRs have been characterized. Class I RNRs are oxygen-dependent enzymes that occur in eubacteria, eukaryotes, and some viruses; class II RNRs are oxygen-independent enzymes confined to bacteria, archaea, and a few unicellular eukaryotes; and class III RNRs are oxygen-sensitive enzymes present in anaerobes. Despite significant differences in their structures and in cofactor requirements, all three enzymes share similar catalytic mechanisms creating a transient cysteinyl radical that initiates the reduction of ribonucleotides, and all employ sophisticated allosteric mechanisms that enable the balanced formation of each of the four deoxyribonucleotides (13, 34).

While eukaryotes in general employ just the class I RNR, many bacteria possess two or even all three RNR classes, the genes of which are typically organized in operons (45) (for a comprehensive listing of RNRs in bacteria, see http://rnrdb.molbio.su.se). *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, and many enterobacteria contain genes encoding three RNRs, class Ia and class Ib RNRs (subdivisions of the class I RNR) and the class III RNR. In *E. coli*, the class Ia RNR operon contains *nrdA* and *nrdB* genes that code for the NrdA catalytic and the NrdB radical-generating subunits, respectively; the class Ib RNR operon consists of four genes, *nrdE* and *nrdF* code for the corresponding subunits NrdE and NrdF, respectively, while *nrdH* codes for NrdH, an ~9-kDa protein that functions as a specific electron donor, and *nrdI* codes for NrdI, an ~15-kDa protein whose function is still unknown. The NrdAB and NrdEF RNRs have limited sequence identity but share many catalytic properties (27). Both require oxygen for generation of a tyrosyl radical stabilized by an iron center, which transfers the radical to an active-site cysteine of NrdA or NrdE. Class III RNRs are encoded by *nrdD*, which occurs in an operon containing *nrdG*, coding for a specific activase that uses *S*-adenosylmethionine to create a stable oxygen-sensitive glycyl radical close to the active site of NrdD. Although considerable information exists regarding the biochemical, structural, and allosteric properties of RNRs (34), our knowledge of the molecular genetic mechanisms by which their differential expression is regulated is more limited.

Aerobic growth of *E. coli* depends on a functional class Ia RNR (23). The aerobic class Ib RNR is not essential for growth, is ordinarily very poorly expressed, and cannot take the place of the class Ia RNR (23). However, when additional copies of the class Ib *nrdHIEF* genes are present on a low-

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copy-number plasmid or an additional copy is inserted into the chromosome, class Ib RNR can support the growth of an *nrdA* temperature-sensitive mutant at the restrictive temperature (24) or an *nrdAB* deletion mutant (16).

The *E. coli* class Ia *nrdAB* RNR genes constitute a tightly regulated transcription unit (19). The operon contains a third nonessential gene, annotated as *yfaE*, that is predicted to encode a ferredoxin-related protein (3). Under aerobic conditions, the DnaA, Fis, and IciA proteins bind to specific sites in the promoter region to regulate *nrdAB* expression, while several *cis*-acting sites and an AT-rich region are important in coupling transcription, in a complex way, to the cell cycle (18, 22). Herrick and Sclavi (21) have recently argued in a comprehensive review that an unknown protein is responsible for controlling cell cycle-dependent *nrdAB* expression. They propose that NrdR, a transcription factor first identified in streptomycetes (6), is a likely candidate to constitute the regulatory link between *nrdAB* expression and nucleotide pool size. The less well-studied *E. coli* class Ib *nrdHIEF* RNR genes form a tight transcription unit in which all four genes are coordinately transcribed from a single promoter (23, 33). Expression of the two sets of RNR genes is dependent on environmental conditions. Under standard laboratory conditions, transcription of *E. coli nrdAB* is much greater than that of *nrdHIEF*. Both *nrdAB* and *nrdHIEF* expression are elevated by DNA damage and by inhibitors of DNA synthesis (15, 23, 33). Hydroxyurea, a free-radical scavenger that inhibits class I RNR enzyme activity (2, 41), induces expression of both *nrdAB* and *nrdHIEF* operons (33). In contrast, *nrdHIEF*, but not *nrdAB*, is highly expressed in response to exposure to oxidative-stress agents (33, 36). Also, *nrdHIEF*, but not *nrdAB*, expression is strongly enhanced in mutants defective in catalase, superoxide dismutase, and alkyl hydroperoxide reductase, which eliminate superoxides and peroxides (33). Furthermore, *nrdHIEF* transcription is highly upregulated in mutants lacking a functional thioredoxin Trx1 or glutaredoxin Grx1, the major reductants of class Ia RNRs (33). Monje-Casas et al. have suggested that the physiological role of the class Ib RNR is primarily a response to oxidative stress, which causes DNA damage (33). However, the mechanism that triggers *nrdHIEF* expression is distinct from either of the known global oxidative-stress regulators, OxyR and SoxRS (43). In addition, the presence of a FUR box in the *nrdHIEF* promoter region suggests that iron metabolism might regulate the transcription of class Ib RNR (31, 48).

Anaerobic growth of *E. coli* requires a functional class III RNR (14). When *E. coli* is shifted from aerobic to microaerophilic or anaerobic growth conditions, *nrdAB* expression is downregulated with concomitant upregulation of *nrdDG* expression. Induction of *nrdDG* transcription is dependent on the FNR system but is independent of the ArcAB two-component system (8). In an *nrdDG* mutant, *nrdAB* expression is upregulated. NrdAB can allow partial growth in the absence of the class III RNR system when traces of oxygen are present, whereas NrdEF cannot, even when the *nrdEF* genes are overexpressed (14). However, the cells grow poorly, have abnormal morphology, and exhibit extensive filamentation. Moreover, deletion of *nrdAB* and *nrdDG* abolishes growth in strict anaerobiosis. Similar findings were reported for *Lactococcus lactis*, which contains class Ib and class III RNRs, an *nrdD* mutant of which was able to grow well under microaerophilic conditions (26). However, when strict anaerobic conditions were applied, growth was dependent on a functional NrdDG (46). These results suggest that while NrdAB and NrdEF both require oxygen, their catalytic activities differ under conditions of limiting oxygen concentration. In contrast, in *Bacillus subtilis*, the class Ib RNR, the only RNR identified in the organism, is essential for both aerobic and anaerobic growth (20).

The recent discovery in *Streptomyces* of a novel regulatory protein, NrdR, that controls RNR gene expression prompted the studies reported here. *Streptomyces coelicolor* contains genes encoding class Ia and class II RNRs that are differentially expressed during vegetative growth (7). Either RNR is sufficient for aerobic growth (6). The *S. coelicolor* NrdR protein is a zinc finger/ATP cone (1) transcriptional regulatory protein (COG1327). Abolishment of NrdR function by a gene knockout resulted in a dramatic increase in transcription of both sets of RNR genes (6). Further studies revealed that NrdR binds to tandem repeat sequences, called NrdR boxes, located in or near to the promoter regions of the class Ia and class II RNR operons (17). NrdR boxes are widespread in diverse bacterial genomes and are almost invariably located in the regulatory regions of different RNR genes (38). In this paper, we explore the role of the *E. coli* homolog of NrdR in regulating the transcription of RNR genes. Although NrdR is thought to regulate the expression of all RNR operons, here, we focus primarily on its effect on control of the aerobic class Ia and class Ib RNR sets of genes.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* K-12 strain MG1655 [F^- λ^- *ilvG rfb-50 rph-1*] is referred to as the wild type. *E. coli* strain XL1 {*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F^- *proAB lacI*^{q} *lacZ* Δ*M15* Tn*10* (Tc^r)]} was used for plasmid constructions; BL21(DE3) [F^- *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3)] was used for protein overexpression.

Culture media and DNA manipulations. *E. coli* strains were grown in Luria-Bertani (LB) medium and supplemented with kanamycin or ampicillin (50 μg/ml and 100 μg/ml, respectively) when appropriate. Plasmid DNA was isolated using the High Pure Plasmid Isolation Kit (Roche). DNA restriction digestions and ligations were carried out according to the manufacturer's instructions. DNA linear fragments were isolated from a 0.9% agarose (Sigma) gel using the QIAquick Gel Extraction Kit (QIAGEN). DNA manipulations were as described previously (39). Electroporation was performed with a Gene Pulsar II apparatus (Bio-Rad Laboratories) according to the manufacturer's instructions.

Cloning of the *nrdR* gene. The *E. coli nrdR* gene was amplified by PCR from MG1655 genomic DNA as described previously (17) using nrdR-For (5′-CATATGCATTGCCCATTCTGTTTCGC) and nrdR-Rev (5′-CTCGAGGTCCTCCAGGCGCGCGATC) as the forward and reverse primers, containing NdeI and XhoI restriction sites, respectively. The PCR-amplified fragment was eluted from an agarose gel, ligated to pGEM-T Easy vector (Promega), and introduced into *E. coli* BL21 by electroporation. Positive transformants were detected by blue-white screening and screened by colony PCR. DNA inserts were sequenced to verify their correctness. The pGEM construct was digested with the restriction endonucleases NdeI and XhoI (Fermentas) and electrophoresed, and the small fragment containing the *nrdR* gene was eluted. The expression vector pET30a(+) (Novagen) was digested with NdeI and XhoI, electrophoresed, and eluted. T4 DNA ligase (Takara) was used to join the two DNA fragments to obtain pET30a(+)::*nrdR*, which was introduced into XL1-Blue by electroporation, and transformants were selected for kanamycin resistance. The *nrdR* DNA insert and the adjacent DNA regions were sequenced to verify the correctness of the construct. The *E. coli nrdR* recombinant protein was expressed with a hexahistidine tag at its C terminus.

Protein overexpression. An overnight culture of *E. coli* BL21(DE3)/pET30a(+)::*nrdR* was diluted in LB medium containing kanamycin (50 μg/ml) to an absorbance of 0.1 at 600 nm and shaken vigorously at 37°C. At an absorbance at 600 nm of 0.6, isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma) was added to a final concentration of 0.4 mM. The cells were incubated for 22 h at 22°C and harvested

by centrifugation at 4,000 × *g* for 20 min at 4°C. The supernatant was discarded, and the cell pellet was stored at −70°C.

Protein purification. Frozen cells were thawed and suspended in sonication buffer: 50 mM Tris-HCl (pH 8.3), 300 mM NaCl, 5 mM imidazole. Phenylmethylsulfonyl fluoride was added to the cell suspension to 1 mM, and the mixture was sonicated in an ultrasonic processor (Misonics) until it was clear. The sonicate was centrifuged at 10,000 × *g* for 45 min at 4°C. The supernatant was loaded on a 5-ml high-capacity Ni^{2+}-CAM resin column (Sigma) equilibrated with the sonication buffer, and the column was washed first with 40 ml sonication buffer, followed by 20 ml buffer containing 15 mM imidazole. Protein was eluted with buffer containing 250 mM imidazole. Protein samples after Ni affinity purification were dialyzed against 50 mM Tris-HCl (pH 8.5), 300 mM NaCl, 5 mM dithiothreitol, 50 μM $ZnCl_2$, 20% glycerol. Recovery of recombinant protein was monitored by the Bradford assay (Bio-Rad) with bovine serum albumin as the standard (9). Purified recombinant proteins were stored at −70°C.

Gel electrophoretic mobility shift assay. Gel electrophoretic mobility shift assays were performed with DNA probes containing the *nrdAB*, *nrdHIEF*, and *nrdDG* promoter regions as described previously (17). Probes were generated by PCR and purified using a PCR purification kit (Roche). They are denoted nrdA (185 bp), nrdH (180 bp), and nrdD (177 bp), respectively, and were designed so that the two NrdR boxes were positioned in the middle of the probes. A control rib probe (190 bp) containing the promoter region of the *rib* operon (which includes *nrdR*) served as a negative control. Mutant nrdA probes, in which the sequence of the NrdR box 1 (upstream box) was changed from 5′-TCACACT ATCTTGCAG to 5′-TgAgACataCaTcCAG or the sequence of the NrdR box 2 (downstream box) was changed from 5′-CCCCTATATATAGTGT to 5′-CgCg TAataAaAcTGT (the changed bases are shown in lowercase letters), were created by an overlap PCR procedure as previously described (17). The changes were made from a comparison of the two 16-bp NrdR box sequences; seven conserved bases were changed to the complementary bases. The primers used in generating wild-type and mutant nrd probes are listed in the supplemental material.

DNA fragments were labeled at the 3′ end with digoxigenin (DIG)-dUTP using the Terminal Transferase kit (Roche, Mannheim, Germany). Binding reactions were carried out in a final volume of 20 μl containing labeled DNA (3 fmol), binding buffer (20 mM Tris-HCl, pH 9.5, 5% [vol/vol] glycerol, 1 mM $MgCl_2$, 40 mM KCl, 1 mM dithiothreitol), purified recombinant NrdR (1 to 15 μg protein), 1 μg salmon sperm DNA, and 0.1 μg bovine serum albumin. Following 30 min of incubation at 37°C, the reaction products were separated on a native 6% polyacrylamide gel in 0.5× Tris-borate buffer, pH 9.5. The gel was contact blotted onto a Hybond N^+ membrane (Amersham Biosciences). Chemiluminescence detection was performed according to the manufacturer's instructions (Roche, Mannheim, Germany). The membrane was exposed to X-ray film (FUJI) for 15 to 40 min at 37°C.

Construction of *E. coli nrdR* mutants. The PCR targeting method of Datsenko and Wanner (12) was used to create deletions in *nrdR*. A 1,371-bp DNA fragment containing the *nrdR* gene with flanking upstream (463-bp) and downstream (484-bp) regions was amplified by PCR from genomic DNA with the forward and reverse primers upR1 (5′-CGGGCAAGGGATCATTCGAC) and downR1 (5′-GTGCGCATCCGCTTGAGAAAGC). The PCR product was cloned into pGEM-T Easy vector to give pGEMT::*nrdR*. The plasmid was digested with HpaI and Bsp119I restriction endonucleases (Fermentas) and ligated to a PCR-amplified Ω2 promoterless and terminatorless kanamycin (*aph-3*) resistance cassette (35) digested with HpaI and Bsp119I enzymes to give pGEMT::Δ*nrdR1*. The PCR product obtained after amplification of pGEMT::Δ*nrdR1* with the upR1 and downR1 primers was electroporated into *E. coli* MG1655 containing plasmid pKD46 (12). Transformants were incubated at 30°C for 2 h in SOC medium (39) supplemented with 10 mM arabinose, and kanamycin-resistant colonies were selected on LB agar plates supplemented with kanamycin. Curing of pKD46 plasmid was effected by overnight incubation at 37°C. The resulting strain was designated MG1655Δ*nrdR1*. The *nrdR1* deletion was verified by PCR and sequencing. Figure 1A shows the positions of the HpaI and Bsp119I restriction sites used to create the 424-bp Δ*nrdR1* deletion in which codons 1 to 134 were replaced by the kanamycin cassette. An *nrdR* deletion mutant lacking the major portion of the ATP cone domain was constructed in a similar way. Plasmid pGEMT::*nrdR* was digested with AjiI and Bsp119I restriction endonucleases and ligated with the HpaI- and Bsp119I-digested kanamycin resistance cassette to give pGEMT::Δ*nrdR2*. Transformants were processed as described above to yield MG1655 Δ*nrdR*2. The *nrdR*2 deletion was verified by PCR and sequencing. Figure 1A shows the positions of the AjiI and Bsp119I restriction sites used to create the 190-bp Δ*nrdR2* deletion, in which codons 57 to 134 were replaced by the kanamycin cassette.

Complementation assay. Complementation was performed using the low-copy-number vector pACYC184 (39) expressing *nrdR*. The *nrdR* gene and its promoter region were amplified from genomic DNA using as primers nrdR-up2 (5′-ATATAGGATCCGGTCGTCCCTTTCTCGTCG) and nrdR-down2 (5′-TA TATGTCGACTAGCGCCCGCGCCATGTAATAC). The PCR product was digested with BamHI and SalI restriction endonucleases (Fermentas) and inserted into the vector digested with the same enzymes. The resulting plasmid, pACYC184::*nrdR*, was introduced into MG1655Δ*nrdR1*.

Genomic-DNA extraction. Genomic DNA was extracted using the Genomic DNA Extraction Kit (QIAGEN) according to the manufacturer's recommendations.

RNA extraction and reverse transcription (RT)-PCRs. For total-RNA preparation, *E. coli* cultures taken at different optical densities at 550 nm (OD_{550}) were mixed directly with RNA Protect Bacteria Reagent (QIAGEN). RNA was extracted using the RNeasy Mini Kit (QIAGEN) according to the manufacturer's recommendations, including the on-column DNase I treatment. To remove further DNA contamination, the eluted RNA was treated with 2 U RNase-free TURBO DNase (Ambion) and kept at −80°C. The amount of RNA was determined from its 260-nm absorption using a Smart SpecTM spectrophotometer (Bio-Rad).

Conversion of RNA to cDNA was performed using a ThermoScript RT-PCR system from Invitrogen. For all RT-PCRs, 1 μg RNA was used and 1 pmol primer (lw; see Table S1 in the supplemental material) was added, together with 2 μl of a 20-pmol deoxynucleoside triphosphate (dNTP) mixture and diethylpyrocarbonate-treated water to a final volume of 12 μl. The RT primers are listed in Table S1 in the supplemental material. The mixture was then incubated at 65°C for 5 min and then transferred to room temperature. To continue the RT reaction, a master mixture was prepared according to the manufacturer's protocol (Invitrogen), added to the RNA tube, and incubated at 50°C for 1 h, followed by a 5-min inactivation step at 85°C in a Robocycler 96 (Stratagene). cDNA was kept at −80°C.

Real-time PCR. Real-time PCR was performed in a 20-μl reaction volume with 1 μl cDNA, TaqMan Universal PCR Master Mix, and TaqMan primers and probes according to the manufacturer's protocol (Applied Biosystems). The detections were performed in a model 7000 ABI PRISM sequence detection system from Applied Biosystems. The primers for real-time PCR probes are listed in Table S1 in the supplemental material. Standard curves for each *nrd* gene were used to analyze the amount of RNA in each sample and in the *gapA* internal control. The amount of target, normalized to the endogenous reference and relative to the calibrator, is given by $2^{-\Delta\Delta C_T}$. In all cases, the values are the mean of three experiments, and the error was below 4%.

Other methods. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described by Laemmli (30). Nucleotide sequencing was determined using an AB1 Prism 3100 genetic analyzer (Applied Biosystems) and the Big Dye terminator cycle sequencing kit (Applied Biosystems) as recommended by the manufacturer.

RESULTS

NrdR differentially regulates transcription of the three *E. coli* RNR operons. The *E. coli* homolog of the *S. coelicolor nrdR* gene is denoted in the genome database as *ybaD* (4). It is also called *ribX* (28), since it is the first gene in an operon (*ybaD-ribD-ribH-nusB-thiL-pgpA*) containing genes determining riboflavin biosynthesis. It codes for a 149-amino-acid protein with a molecular mass of 17.2 kDa. It shares 43% identity (65% similarity) in a 149-amino-acid overlap with the *S. coelicolor* NrdR protein and is predicted to possess similar zinc finger and ATP cone domains. We purified the *E. coli* YbaD protein and showed that it binds zinc and contains ATP/dATP, as previously reported for the streptomycete NrdR protein (data not presented). Henceforth, we refer to the *E. coli* YbaD as NrdR. Figure 1A schematically shows the organization of the *E. coli nrdR-rib* operon. Figure 1B shows the nucleotide and amino acid sequences of the *E. coli* NrdR and the positions of the zinc finger and ATP cone domains.

To establish that *nrdR* is expressed during growth, total RNA was isolated from cells at the early exponential phase

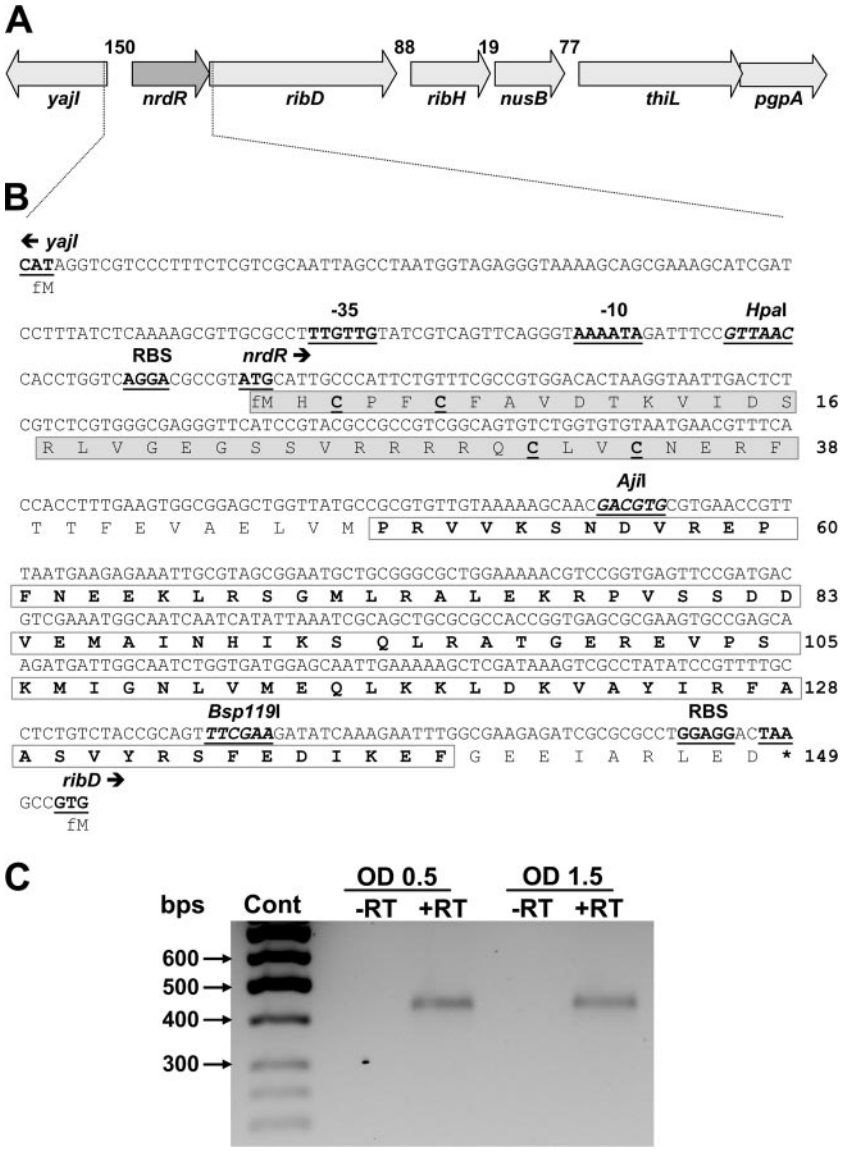

FIG. 1. Structure and expression of the *E. coli nrdR* operon. (A) Organization of the genes. The *nrdR* operon comprises *nrdR* (*ybaD*), coding for NrdR, a transcriptional regulator of RNR genes; *ribD*, coding for the bifunctional diaminohydroxyphosphoribosylaminopyrimidine deaminase/5-amino-6-(5-phosphoribosylamino) uracil reductase; *ribH*, coding for the beta subunit of riboflavin synthase or 6,7-dimethyl-8-ribityllumazine synthase; *nusB*, coding for the transcription antitermination factor NusB; *thiL*, coding for thiamine-monophosphate kinase; and *pgpA*, coding for phosphatidylglycerophosphatase A. The arrows indicate the orientations of genes. (B) Nucleotide sequence of the *nrdR* regulatory region and structural gene. The deduced NrdR amino acid sequence is shown. The predicted zinc finger DNA binding motif and ATP cone motif are shown in shaded and open boxes, respectively. Predicted promoter −10 and −35 recognition elements, ribosomal binding site (RBS), and translational initiation and termination codons are shown in boldface. The arrows indicate the directions of transcription of genes. The restriction sites used in the construction of *nrdR* deletion mutants (see Materials and Methods) are shown boldface italics and underlined. (C) Semiquantitative RT-PCR analysis of *nrdR* at exponential (OD = 0.5) and early stationary (OD = 1.5) phases of growth. RT, reverse transcriptase.

(OD_{550} = 0.5) and early stationary phase (OD_{550} = 1.5), reverse transcribed with primers specific for *nrdR*, and analyzed by semiquantitative RT-PCR. Figure 1C shows that *nrdR* is transcribed at similar levels during growth. To determine whether *E. coli* NrdR regulates expression of the different *nrd* genes, we initially used the Targetron Gene Knockout System (Sigma-Aldrich) to disrupt the *nrdR* gene by insertion of a group II intron conferring kanamycin resistance. The insertion mutation caused a pronounced drop in the growth rate compared to the wild type, presumably due to a strong polar effect on transcription of the downstream *ribD*, *ribH*, *nusB*, *thiL*, and *pgpA* genes (data not shown). We therefore created deletions in *nrdR* in which the zinc finger and ATP cone domains (MG1655Δ*nrdR1*) or the ATP cone domain alone (MG1655Δ*nrdR*2) were replaced by a kanamycin cassette without altering the transcription of the downstream *nrdR* genes (see Material and Methods). Both *nrdR* deletion-substitution mutants were indistinguishable in their growth on LB broth and minimal medium from the parent strain (data not shown).

To assess the effect of NrdR on the transcription of *nrdAB*

TABLE 1. Amounts of *nrdA*, *nrdE*, *nrdD*, and *nrdR* RNAs present in *E. coli* MG1655 during growth[a]

OD_{550}	RNA amt (pg/μg total RNA)			
	nrdA	*nrdE*	*nrdD*	*nrdR*
0.4	21.7	0.75	0.55	19.6
0.8	24.8	0.05	1.4	22.8
1.3	5.2	0.06	0.63	10.2
1.6	5.8	0.02	0.74	10.2

[a] MG1655 is the wild-type strain used in this work. The numbers are the mean values of three replicates from two independent experiments. The variation between the numbers was less than 4%.

and *nrdHIEF* genes, quantitative measurements of specific RNA levels were made in the wild type and in *nrdR* mutants using real-time RT-PCR. The *nrdA* and *nrdE* genes served as the respective target sequences for the aerobic RNR genes. In parallel, measurements were made of RNA levels of the class III *nrdDG* RNR genes using *nrdD* as the target sequence, even though NrdD is functional only in anaerobic growth. Table 1 shows the amounts of *nrdA*, *nrdE*, and *nrdD* RNAs present in wild-type and mutant strains determined at what are conveniently defined as the early (OD_{550} = 0.4), mid-exponential (OD_{550} = 0.8), late exponential (OD_{550} = 1.3), and early stationary (OD_{550} = 1.6) phases of growth. In MG1655 (wild type), the amount of *nrdA* RNA decreased three- to fourfold as the culture progressed from the early exponential to the early stationary phase (Table 1). The fall in *nrdA* RNA levels presumably reflects the decrease in need for new enzyme synthesis as cells enter the later phases of growth. In the case of *nrdE*, the decrease in RNA levels in MG1655 during growth is much greater. The amount of *nrdE* RNA at the early exponential phase is some 30-fold less than that of *nrdA* RNA at the same growth phase. At later growth phases, the amount of *nrdE* drops dramatically to several hundredfold less than that of *nrdA* at the same growth phases. In the case of *nrdD*, the changes in RNA levels during growth are modest and are less than a twofold increase. Interestingly, the amounts of *nrdD* RNA relative to *nrdA* RNA at the early exponential and early stationary phases increased by fivefold. Comparison of the actual amounts of *nrdA*, *nrdE*, and *nrdD* RNAs in MG1655 (Table 1) shows clearly that *nrdAB* transcripts were far more abundant than *nrdHIEF* and *nrdDG* transcripts at all times during growth. Measurements of the amount of *nrdR* RNA in MG1655 showed a twofold overall drop in level during the growth cycle (Table 1).

Figure 2 presents the results in terms of the induction factors for *nrdA* (top), *nrdE* (middle), and *nrdD* (bottom). The induction factors for the two mutant strains, MG1655Δ*nrdR1* and MG1655Δ*nrdR2*, are the ratios of the amounts of specific RNAs made in these strains to that made in MG1655 (wild type) at the same growth phase. The induction values for MG1655 at each growth phase were arbitrarily set as 1.0. In the MG1655Δ*nrdR1*mutant (which is principally an *nrdR* null mutant), the level of *nrdA* RNA changed little during growth. At the early exponential phase, the *nrdA* induction factor (the *nrdA* change compared to that of the wild type at the same growth phase) was about 1.5-fold more than that of the wild type at the same growth phase. Subsequently, the induction factor increased and reached about fourfold higher at the early stationary phase. Similar increases occurred in the *nrdA* induction factor in the MG1655Δ*nrdR2* mutant (which lacks only the ATP cone domain). The induction factor was 0.75 at the early exponential phase and increased to some eightfold at the early stationary phase.

In contrast to *nrdA*, much more pronounced changes occur in *nrdE* expression in the two *nrdR* mutant strains. In MG1655Δ*nrdR1*, the *nrdE* induction value (the *nrdE* change compared to that of the wild type at the same growth phase) at the early exponential phase was some 4.6-fold more than that of the wild type and markedly increased during growth. It was some 22-fold higher at the mid-exponential, 50-fold higher at the late exponential, and 26-fold higher at the early stationary phases of growth. In MG1655Δ*nrdR2*, the changes were even more striking. The *nrdE* induction value was about 7-fold higher than that in the wild type at the early exponential growth phase, 178-fold higher at the mid-exponential phase, and 70- to 75-fold higher at the late exponential and early stationary phases of growth. To confirm that induction of *nrdE* expression in the Δ*nrdR1* deletion strain is a consequence of abrogation of NrdR function, we introduced a pACYC184 low-copy-number plasmid bearing the intact *nrdR* gene and its regulatory region into MG1655Δ*nrdR1*. Semiquantitative RT-PCR analysis showed that the plasmid expresses *nrdR* and causes down regulation of *nrdE* to the level present in the wild type (data not shown). The impressive increases in *nrdE* expression in the two *nrdR* mutant strains over that of the wild type from the mid-exponential stage of growth reflect in part the very low *nrdE* RNA levels present in the wild type at these stages. As can be seen from Table 1 (and the supplemental material), which reports absolute amounts of *nrdA* and *nrdE* RNAs, the class Ia RNR operon is clearly the dominant RNR operon expressed throughout aerobic growth, and the actual amounts of *nrdE* RNA are 5- to 50-fold less than those of *nrdA* throughout the growth cycle.

We also monitored the levels of *nrdD* transcripts, coding for the NrdD anaerobic RNR, in wild-type and mutant strains. Induction levels (the *nrdD* change compared to that of the wild type at the same growth phase) in MG1655Δ*nrdR1* and MG1655Δ*nrdR2* were about seven- and fourfold higher at the early exponential phase, respectively. At the later growth phases, the induction values in MG1655Δ*nrdR1* were 4- to 6-fold higher, and they were 5- to 10-fold higher in MG1655Δ*nrdR2*. However, the actual amounts of *nrdD* RNA were 4- to 10-fold less than those of *nrdA* RNA (see the supplemental material).

NrdR binds to the promoter regions of all three *E. coli* RNR operons. On the basis of previous studies that showed that NrdR regulates the transcription of the *S. coelicolor* class Ia and class II RNR operons and binds to their promoter regions, we supposed that NrdR might control the expression of the *E. coli* RNR genes in a similar fashion. NrdR is a zinc finger/ATP cone protein that binds to tandem 16-bp repeat sequences, termed NrdR boxes, located almost invariably in front of RNR genes (17, 38). The 5′ upstream regions of all three *E. coli* RNR operons contain two tandem NrdR boxes in their promoter regions (38). Figure 3 shows an alignment of the *E. coli* and *S. enterica* serovar Typhimurium *nrdAB*, *nrdHIEF*, and *nrdDG* promoter regions. The NrdR boxes and the positions of the −10 and −35 promoter elements and transcription start sites, where known, are indicated. The alignment reveals that

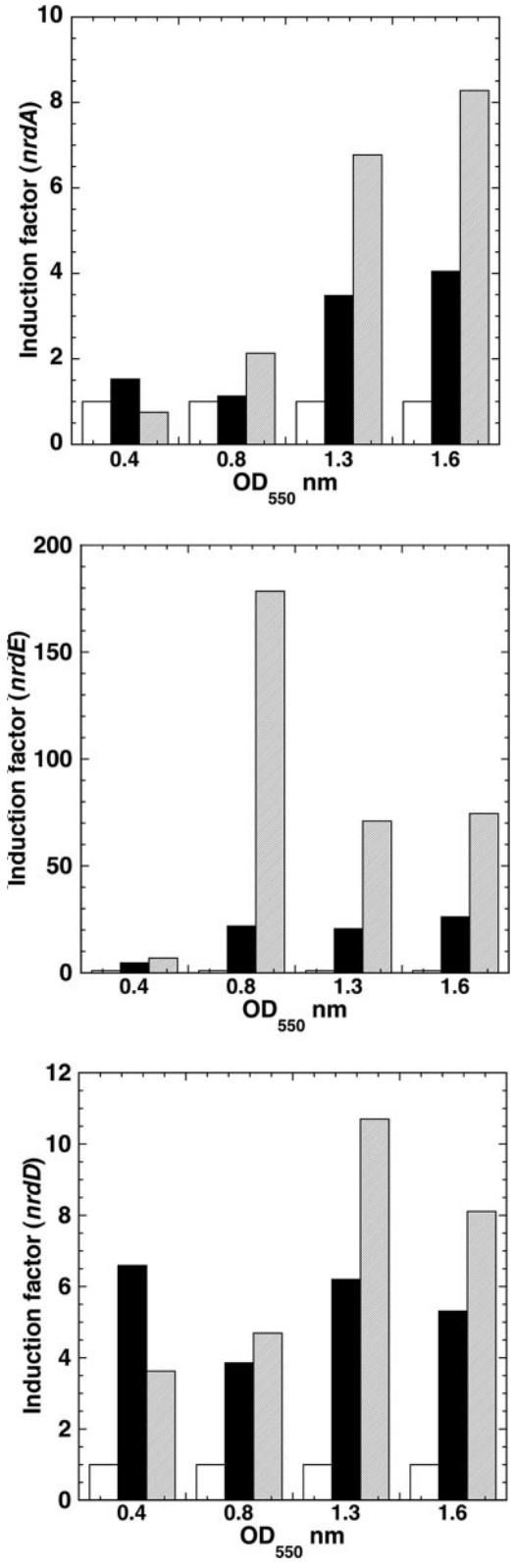

FIG. 2. Real-time RT-PCR analysis of *nrdAB*, *nrdHIEF*, and *nrdDG* in MG1655 (wild type), MG1655Δ*nrdR1*, and MG1655Δ*nrdR2* RNAs as a function of the growth phase. Real-Time PCR measurements were performed using TaqMan primers and probes, and detection was performed in a model 7000 ABI Prism Sequence Detection System from Applied Biosystems. The induction factors in the two mutant strains are the ratios of the amount of specific RNA in the mutant strain to that in the wild type at the same OD normalized with the endogenous reference (*gapA*). (Top) nrdA probe. (Middle) nrdE probe. (Bottom) nrdD probe. Open bars, MG1655 wild type; black bars, MG1655Δ*nrdR1*; gray bars, MG1655Δ*nrdR2*.

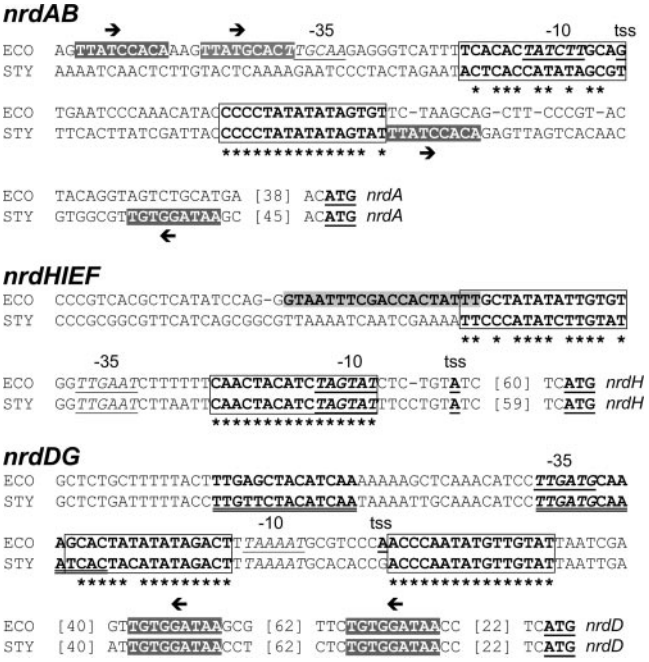

FIG. 3. Alignment of the *E. coli* (ECO) and *S. enterica* serovar Typhimurium (STY) *nrdAB*, *nrdHIEF*, and *nrdDG* regulatory regions showing the positions and sequences of the promoter elements and NrdR boxes. NrdR box motifs are aligned and are shown in boldface and enclosed in rectangular boxes. Identical bases in motifs are indicated by asterisks. The reported experimentally determined promoter −10 and −35 recognition elements are shown (8, 25, 47) in italics and underlined. DnaA box motifs are shown in boldface in white letters in gray boxes, and their relative orientations are indicated by arrows. A FUR box located upstream of the *nrdHIEF* genes is shown in boldface in a gray box. Two FNR box motifs in the upstream region of *nrdD* are shown in boldface and doubly underlined. Other symbols shown in boldface are tss, the transcriptional start site (where known), and the translational start codon.

(i) the positions of the NrdR boxes in the *nrdAB*, *nrdHIEF*, and *nrdDG* regulatory regions differ with respect to the positions of their promoter elements; (ii) the sequences of NrdR boxes belonging to the same class of RNR are significantly more alike than the sequences of NrdR boxes from different RNRs of the same organism; and (iii) the proximal NrdR box 2 sequences are more conserved than the distal NrdR box 1 sequences.

To assess whether NrdR binds to the 5′ upstream regions of the *E. coli* RNR operons, we performed gel electrophoretic mobility shift assays with purified NrdR and PCR-amplified DIG-labeled ~180-bp DNA probes that spanned the two NrdR boxes. The probes, denoted nrdA (185 bp), nrdH (180 bp), and nrdD (177 bp), were designed so that the two boxes were positioned in the central portion of the probe. Figure 4A shows that NrdR binds to all three probes. The relatively large

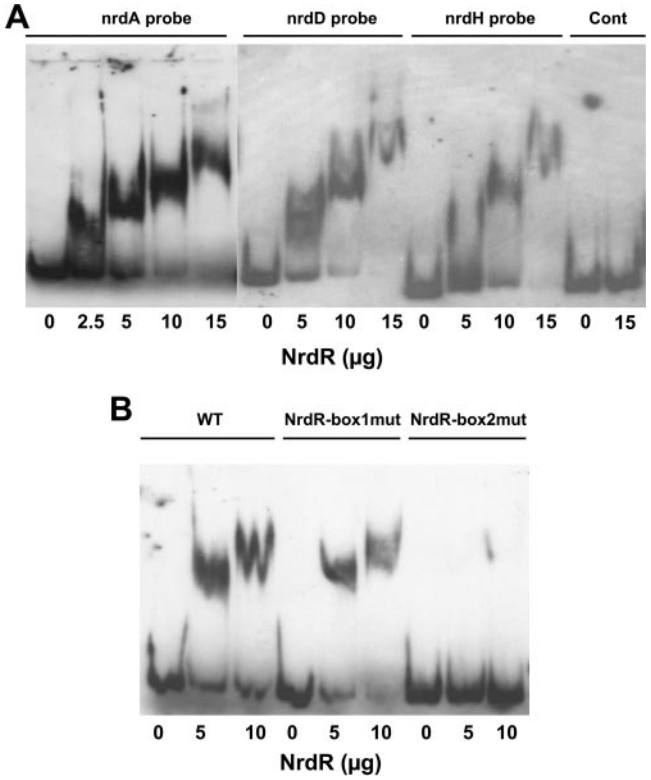

FIG. 4. NrdR binding to the DNA regulatory region of the *E. coli nrdAB*, *nrdHIEF*, and *nrdDG* operons. DIG labeling of DNA probes and gel electrophoretic mobility shift assays were performed as described in Materials and Methods. (A) nrdA probe with 0, 2.5, 5, 10, and 15 μg protein; nrdD probe with 0, 5, 10, and 15 μg protein; nrdH probe with 0, 5, 10, and 15 μg protein; and control (Cont) rib probe with 0 and 15 μg protein. (B) NrdR binding to DNA of nrdA wild-type and mutant NrdR box 1 and box 2 probes. The NrdR box1 sequence was changed from 5′-TCACACTATCTTGCAG to 5′-TgAgACataCa TcCAG; the sequence of NrdR box 2 was changed from 5′-CCCCTA TATATAGTGT to 5′-CgCgTAataAaAcTGT. The seven changes are shown in lowercase letters. Wild-type (WT) probe with 0, 5, and 10 μg protein; mutant (mut) NrdR box 1 probe with 0, 5, and 10 μg protein; mutant NrdR box 2 probe with 0, 5, and 10 μg protein.

amounts of protein used in the gel shift experiments and the appearance of multiple DNA-NrdR complexes probably reflect the tendency of NrdR to aggregate into oligomeric forms (17). NrdR did not bind to a control DNA probe consisting of the upstream regulatory region of the *rib* operon at any of the NrdR concentrations used.

To assess the roles of the two NrdR boxes, NrdR was tested for binding to two mutant nrdA probes, one containing a mutant NrdR box 1 and another containing a mutant NrdR box 2. Figure 4B shows that NrdR was unable to bind to the NrdR box 2 probe, in which the wild-type sequence, 5′-CCCC TATATATAGTGT, was changed to 5′-CgCgTAataAaAc TGT. In contrast, NrdR was able to bind to the mutant NrdR box 1 probe, in which the wild-type sequence, 5′-TCACACT ATCTTGCAG, was changed to 5′-TgAgACataCaTcCAG (the changed bases are shown in lowercase letters). This result indicates that the proximal NrdR box 2, the more highly conserved of the two sequences (Fig. 3), is more important for NrdR binding than box 1.

DISCUSSION

The *E. coli ybaD* gene codes for a protein, YbaD, annotated in the genome databases as of unknown function. It is highly conserved throughout bacteria and generally absent in archaea. Previous reports on YbaD indicated a complex phenotype; it was found to be nonessential, since deletion of *ybaD* in a collection of single-gene in-frame mutations did not cause nonviability (3); it occurs as a partner of several interacting protein systems (10); it is a thioredoxin TrxA-targeted protein (29); and it belongs to an uber-operon predicted to be functionally or transcriptionally related to the class Ib RNR operon (11). *E. coli* YbaD is homologous to the *Streptomyces* NrdR transcriptional regulator, and we have denoted it NrdR in this work. The *S. coelicolor* NrdR is not essential for growth and acts to repress transcription of the class Ia and class II RNR genes (6). The studies reported here show that *E. coli* NrdR is not essential and that it represses transcription of *nrdHIEF* but has a much smaller effect on transcription of *nrdAB* and *nrdDG*. It is noteworthy that despite the impressive increase in *nrdHIEF* transcription in mutants lacking NrdR (up to 180-fold derepression), the class Ia RNR operon is the dominant class I RNR operon that is expressed aerobically in LB medium, with 5- to 50-fold-higher absolute transcript levels. We also show that NrdR binds to the *nrdAB*, *nrdHIEF*, and *nrdDG* 5′ untranslated regulatory regions. We propose that NrdR differentially regulates *nrdAB*, *nrdHIEF*, and *nrdD* transcription in aerobic growth by binding to NrdR boxes in the promoter regions to alter promoter activity. How does this occur?

One possible mechanism to account for differential expression of class I RNR genes assumes that NrdR repression depends on the position of the NrdR boxes with respect to the *nrdAB* and *nrdHIEF* promoters. Inspection of the regulatory regions of the *E. coli nrdAB* and *nrdHIEF* (and *nrdDG*) operons shows that they contain tandem repeat sequences that essentially conform to the consensus NrdR box motif, acaCw AtATaTwGtgt (38). The two 16-bp repeats are separated by 15 bp, and a similar organization occurs in the corresponding regulatory regions of the *S. enterica* serovar Typhimurium RNR operons. One of the sequences (NrdR box 2) was first noted in a comparison of the *E. coli* and *S. enterica* serovar Typhimurium promoter regions (25). It was also identified in *E. coli* as a conserved motif, termed Even 35, by phylogenetic footprinting of orthologous *nrdA* and *nrdD* genes in closely related species (37). The positions of the NrdR boxes with respect to the *nrdAB* and *nrdHIEF* promoter elements differ (Fig. 3). In the *nrdAB* promoter region, the two NrdR boxes are located downstream of the −35 promoter recognition element, with NrdR box 1 overlapping the −10 promoter element and the transcription start site. In the *nrdHIEF* promoter region, the two NrdR boxes bracket the −35 promoter recognition element, with NrdR box 2 overlapping the −10 promoter element. A similar arrangement occurs in the promoter regions of the *S. enterica* serovar Typhimurium RNR operons. We suppose that NrdR may be more effective in inhibiting *nrdHIEF* transcription than *nrdAB* because it more likely blocks the binding of RNA polymerase to the *nrdHIEF* promoter than to the *nrdAB* promoter. This view is consistent with the finding that NrdR box 2, but not NrdR box 1, is necessary for NrdR binding (Fig. 4B) and that the position of NrdR box

2 in *nrdHIEF* overlaps with the −10 promoter element, whereas in *nrdAB* it is located 16 bp downstream of the −10 element (Fig. 3). A similar mechanism was proposed to account for the differential effect of NrdR on transcription of the *S. coelicolor nrdAB* and *nrdRJ* genes, where the NrdR boxes either overlap with the promoter elements or are located just upstream, respectively (17).

Alternatively, differential regulation could be a consequence of differences in the sequences of the NrdR box motifs. Comparison of the sequences of the *E. coli* and *S. enterica* serovar Typhimurium *nrdAB* NrdR boxes reveals high conservation; NrdR box 1 shares 9/16 identities, while NrdR box 2 is identical in all but one of the 16 bases (Fig. 3) A similar picture emerges from a comparison of the *E. coli* and *S. enterica* serovar Typhimurium *nrdHIEF* and *nrdDG* NrdR box sequences. In contrast, sequence conservation between NrdR boxes belonging to *nrdAB*, *nrdHIEF*, and *nrdDG* promoter regions is less pronounced. The greater conservation in box 2 suggests that it may be the primary site for NrdR binding (all three probes showed similar NrdR binding profiles). Indeed, preliminary studies in which the two *nrdAB* NrdR box sequences were mutated showed that box 2 is critical for binding (Fig. 4B). Although NrdR was found to bind in vitro to *nrdAB* and *nrdHIEF* (and *nrdDG*) promoter probes (Fig. 4A), differences in the positions and extents of binding could have a significant influence on promoter activity. Possibly, differences in both the positions and sequences of NrdR boxes may account, at least in part, for differential effects on transcription of *nrdAB* and *nrdHIEF*. Further studies will be required to determine the correctness of these views. Nevertheless, it is of interest that deletions in the region immediately downstream of the *nrdAB* transcription start site were reported to cause a 10-fold increase in expression (47). We note that the deletion removes the downstream NrdR box 2, supporting the view that NrdR controls transcription via its interaction with NrdR box motifs.

The effect of NrdR on transcription of the class III anaerobic RNR genes in aerobic growth is unexpected. In the wild type, the level of *nrdD* transcription did not change appreciably during the growth cycle. In the *nrdR* mutants, *nrdD* transcription increased by four- to eightfold throughout growth. Expression of the *nrdDG* genes occurs under oxygen-limiting conditions, during anaerobiosis, and, to a lesser extent, in the deceleration phase of aerobically growing cultures and is completely dependent on the global transcription regulator FNR, but not on the ArcA-ArcB two-component system (8). The results presented here suggest that induction of *nrdDG* expression is, in part, controlled by NrdR. Sun et al. (44) identified an FNR binding sequence in the upstream region of *nrdD*, and Boston and Atlung (8) noted a second site; one site is centered at position −65 relative to the transcription start site, and the second overlaps the −35 promoter sequence. The latter FNR site also overlaps with the upstream NrdR box 1 sequence. Hence, the *E. coli* class III *nrdDG* operon appears to be under positive regulation by FNR and negative regulation by NrdR.

Numerous environmental factors are known to influence expression of the *E. coli* class Ia and class Ib RNR aerobic genes, including oxygen tension, inhibition of DNA synthesis, DNA-damaging agents, nutritional status, and oxidative- and thiol-redox stress agents (21). The coordination of DNA replication with cell growth involves multiple levels of regulation (reviewed in reference 21). Gon and coworkers have recently proposed a model in which the nucleotide bound state of *E. coli* DnaA regulates transcription of the *nrdAB* genes (16). In addition to its role in initiating DNA replication, DnaA is a transcriptional regulator that controls positively or negatively the expression of *nrdAB* by binding to DnaA boxes immediately upstream of the *nrdAB* operon (32, 42). In their model, ATP-DnaA acts as a repressor of *nrdAB* transcription; after initiation of DNA replication, ATP DnaA is converted to ADP DnaA, resulting in increased *nrdAB* expression and thereby coordinating DNA replication and RNR synthesis during the cell cycle. Class Ib *nrdEF* genes would not be subject to this regulatory mode, since the promoter region lacks a DnaA binding site. Gon and coworkers have proposed an additional control mechanism to regulate NrdAB activity in which an excess of dNTPs leads to binding of dNTP by DnaA and repression of *nrdAB* expression (16). This is consistent with findings that DnaA binds dATP (40). Thus, high dNTP levels may negatively control expression of the class Ia *nrdAB* genes by transcriptional repression and, because NrdA contains a regulatory site that binds dATP, negatively control enzyme activity by allosteric regulation. Class Ib RNRs lack this allosteric regulatory site. Hence, class Ia RNRs would provide protection against possible harmful effects of overproduction of dNTPs by negative feedback, while class Ib RNRs may serve to enable synthesis of elevated levels of dNTPs under conditions where higher levels are needed, such as in DNA repair.

In this communication, we have described a new mechanism for controlling expression of the *E. coli nrdAB* and *nrdHIEF* genes during aerobic growth via binding of the transcriptional repressor NrdR to specific motifs in the promoter regions. The physiological role of the *nrdHIEF* operon in *E. coli* is not clear, since under aerobic conditions, NrdAB is the major source of the class I RNR and NrdEF is made in insufficient amounts to be able to replace it for growth. Nevertheless, it was reported that an extra chromosomal copy of *nrdHIEF* (or low-copy-number *nrdHIEF* plasmids) suffice to enable growth of an *nrdA* mutant, with the extra copy dramatically increasing the *nrdHIEF* level (16, 23). Recent observations suggest a possible new function for class Ib RNR under iron limitation. A Fur box was described in the region immediately upstream of *nrdHIEF* (Fig. 3) (48). Global transcription analysis of an *E. coli fur* mutant showed that *nrdHIEF* was partially derepressed (31). These observations imply the existence of a finely tuned regulatory switch that controls *nrdHIEF* expression. This is also evident in the marked changes in *nrdHIEF* expression found in the wild type during growth and other reports of the dependence of *nrdHIEF* expression on the growth phase and growth medium (33). The studies reported here suggest that the cellular level of NrdR is ordinarily sufficient to repress *nrdHIEF*, but not *nrdAB*, expression but is insufficient to repress additional *nrdHIEF* copies. This view is consistent with the finding that abolishing NrdR function caused drastic increases in *nrdHIEF* expression. These results suggest that induction of the *E. coli nrdHIEF* operon is likely to be physiologically important under conditions in which *nrdAB* expression is inactive or severely limited and possibly under conditions, such as in infection, when iron availability may be important.

NrdR, like DnaA, binds ATP and dATP and controls the transcription of RNR genes by binding to specific target sites in

the promoter regions. Elsewhere, it was shown in *S. coelicolor* that NrdR binds to these motifs via the N-terminal zinc finger domain and that efficient NrdR binding is dependent on the presence of an intact ATP cone domain that binds ATP/dATP (17). Plausibly, the intracellular level of ATP/dATP modulates NrdR binding and enables differential regulation of *nrdAB* and *nrdHIEF* transcription. As noted above, dATP also binds DnaA, and the *nrdAB* operon contains two tandem DnaA boxes proximal to NrdR box 1. Thus, NrdR and DnaA may both function, in concert or otherwise, as dNTP-dependent repressors of RNR expression. Finally, we note that because ATP/dATP allosterically regulate the class Ia RNR, but not the class Ib RNR, enzyme activity, ATP/dATP may play a dual role in controlling transcription, as well as enzyme activity.

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REFERENCES

1. **Aravind, L., Y. I. Wolf, and E. V. Koonin.** 2000. The ATP-cone: an evolutionarily mobile, ATP-binding regulatory domain. J. Mol. Microbiol. Biotechnol. **2:**191–194.
2. **Atkin, C. L., L. Thelander, P. Reichard, and G. Lang.** 1973. Iron and free radical in ribonucleotide reductase. Exchange of iron and Mossbauer spectroscopy of the protein B2 subunit of the *Escherichia coli* enzyme. J. Biol. Chem. **248:**7464–7472.
3. **Baba, T., T. Ara, M. Hasegawa, Y. Takai, Y. Okumura, M. Baba, K. A. Datsenko, M. Tomita, B. L. Wanner, and H. Mori.** 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol. Syst. Biol **2:**2006.0008.
4. **Blattner, F. R., G. Plunkett III, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, J. Collado-Vides, J. D. Glasner, C. K. Rode, G. F. Mayhew, J. Gregor, N. W. Davis, H. A. Kirkpatrick, M. A. Goeden, D. J. Rose, B. Mau, and Y. Shao.** 1997. The complete genome sequence of *Escherichia coli* K-12. Science **277:**1453–1474.
5. **Borovok, I., B. Gorovitz, R. Schreiber, Y. Aharonowitz, and G. Cohen.** 2006. Coenzyme B12 controls transcription of the *Streptomyces* class Ia ribonucleotide reductase *nrdABS* operon via a riboswitch mechanism. J. Bacteriol. **188:**2512–2520.
6. **Borovok, I., B. Gorovitz, M. Yanku, R. Schreiber, B. Gust, K. Chater, Y. Aharonowitz, and G. Cohen.** 2004. Alternative oxygen-dependent and oxygen-independent ribonucleotide reductases in *Streptomyces*: cross-regulation and physiological role in response to oxygen limitation. Mol. Microbiol. **54:**1022–1035.
7. **Borovok, I., R. Kreisberg-Zakarin, M. Yanko, R. Schreiber, M. Myslovati, F. Aslund, A. Holmgren, G. Cohen, and Y. Aharonowitz.** 2002. *Streptomyces* spp. contain class Ia and class II ribonucleotide reductases: expression analysis of the genes in vegetative growth. Microbiology **148:**391–404.
8. **Boston, T., and T. Atlung.** 2003. FNR-mediated oxygen-responsive regulation of the *nrdDG* operon of *Escherichia coli*. J. Bacteriol. **185:**5310–5313.
9. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
10. **Butland, G., J. M. Peregrin-Alvarez, J. Li, W. Yang, X. Yang, V. Canadien, A. Starostine, D. Richards, B. Beattie, N. Krogan, M. Davey, J. Parkinson, J. Greenblatt, and A. Emili.** 2005. Interaction network containing conserved and essential protein complexes in *Escherichia coli*. Nature **433:**531–537.
11. **Che, D., G. Li, F. Mao, H. Wu, and Y. Xu.** 2006. Detecting uber-operons in prokaryotic genomes. Nucleic Acids Res. **34:**2418–2427.
12. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA **97:**6640–6645.
13. **Eriksson, M., U. Uhlin, S. Ramaswamy, M. Ekberg, K. Regnström, B. M. Sjöberg, and H. Eklund.** 1997. Binding of allosteric effectors to ribonucleotide reductase protein R1: reduction of active-site cysteines promotes substrate binding. Structure **5:**1077–1092.
14. **Garriga, X., R. Eliasson, E. Torrents, A. Jordan, J. Barbé, I. Gibert, and P. Reichard.** 1996. *nrdD* and *nrdG* genes are essential for strict anaerobic growth of *Escherichia coli*. Biochem. Biophys. Res. Commun. **229:**189–192.
15. **Gibert, I., S. Calero, and J. Barbé.** 1990. Measurement of in vivo expression of *nrdA* and *nrdB* genes of *Escherichia coli* by using *lacZ* gene fusions. Mol. Gen. Genet. **220:**400–408.
16. **Gon, S., J. E. Camara, H. K. Klungsoyr, E. Crooke, K. Skarstad, and J. Beckwith.** 2006. A novel regulatory mechanism couples deoxyribonucleotide synthesis and DNA replication in *Escherichia coli*. EMBO J. **25:**1137–1147.
17. **Grinberg, I., T. Shteinberg, B. Gorovitz, Y. Aharonowitz, G. Cohen, and I. Borovok.** 2006. The *Streptomyces* NrdR transcriptional regulator is a Zn ribbon/ATP cone protein that binds to the promoter regions of class Ia and class II ribonucleotide reductase operons. J. Bacteriol. **188:**7635–7644.
18. **Han, J. S., H. S. Kwon, J. B. Yim, and D. S. Hwang.** 1998. Effect of IciA protein on the expression of the *nrd* gene encoding ribonucleoside diphosphate reductase in *E. coli*. Mol. Gen. Genet. **259:**610–614.
19. **Hanke, P. D., and J. A. Fuchs.** 1983. Regulation of ribonucleoside diphosphate reductase mRNA synthesis in *Escherichia coli*. J. Bacteriol. **154:**1040–1045.
20. **Härtig, E., A. Hartmann, M. Schätzle, A. M. Albertini, and D. Jahn.** 2006. The *Bacillus subtilis nrdEF* genes, encoding a class Ib ribonucleotide reductase, are essential for aerobic and anaerobic growth. Appl. Environ. Microbiol. **72:**5260–5265.
21. **Herrick, J., and B. Sclavi.** 2007. Ribonucleotide reductase and the regulation of DNA replication: an old story and an ancient heritage. Mol. Microbiol. **63:**22–34.
22. **Jacobson, B. A., and J. A. Fuchs.** 1998. Multiple cis-acting sites positively regulate *Escherichia coli nrd* expression. Mol. Microbiol. **28:**1315–1322.
23. **Jordan, A., E. Aragall, I. Gibert, and J. Barbé.** 1996. Promoter identification and expression analysis of *Salmonella typhimurium* and *Escherichia coli nrdEF* operons encoding one of two class I ribonucleotide reductases present in both bacteria. Mol. Microbiol. **19:**777–790.
24. **Jordan, A., I. Gibert, and J. Barbé.** 1994. Cloning and sequencing of the genes from *Salmonella typhimurium* encoding a new bacterial ribonucleotide reductase. J. Bacteriol. **176:**3420–3427.
25. **Jordan, A., I. Gibert, and J. Barbé.** 1995. Two different operons for the same function: comparison of the *Salmonella typhimurium nrdAB* and *nrdEF* genes. Gene **167:**75–79.
26. **Jordan, A., E. Pontis, F. Åslund, U. Hellman, I. Gibert, and P. Reichard.** 1996. The ribonucleotide reductase system of *Lactococcus lactis*. Characterization of an NrdEF enzyme and a new electron transport protein. J. Biol. Chem. **271:**8779–8785.
27. **Jordan, A., E. Pontis, M. Atta, M. Krook, I. Gibert, J. Barbé, and P. Reichard.** 1994. A second class I ribonucleotide reductase in *Enterobacteriaceae*: characterization of the *Salmonella typhimurium* enzyme. Proc. Natl. Acad. Sci. USA **91:**12892–12896.
28. **Kasai, S., and T. Sumimoto.** 2002. Stimulated biosynthesis of flavins in *Photobacterium phosphoreum* IFO 13896 and the presence of complete rib operons in two species of luminous bacteria. Eur. J. Biochem. **269:**5851–5860.
29. **Kumar, J. K., S. Tabor, and C. C. Richardson.** 2004. Proteomic analysis of thioredoxin-targeted proteins in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **101:**3759–3764.
30. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
31. **McHugh, J. P., F. Rodriguez-Quinones, H. Abdul-Tehrani, D. A. Svistunenko, R. K. Poole, C. E. Cooper, and S. C. Andrews.** 2003. Global iron-dependent gene regulation in *Escherichia coli*. A new mechanism for iron homeostasis. J. Biol. Chem. **278:**29478–29486.
32. **Messer, W., and C. Weigel.** 1997. DnaA initiator—also a transcription factor. Mol. Microbiol. **24:**1–6.
33. **Monje-Casas, F., J. Jurado, M. J. Prieto-Alamo, A. Holmgren, and C. Pueyo.** 2001. Expression analysis of the *nrdHIEF* operon from *Escherichia coli*. Conditions that trigger the transcript level in vivo. J. Biol. Chem. **276:**18031–18037.
34. **Nordlund, P., and P. Reichard.** 2006. Ribonucleotide reductases. Annu. Rev. Biochem. **75:**681–706.
35. **Perez-Casal, J., M. G. Caparon, and J. R. Scott.** 1991. Mry, a *trans*-acting positive regulator of the M protein gene of *Streptococcus pyogenes* with similarity to the receptor proteins of two-component regulatory systems. J. Bacteriol. **173:**2617–2624.
36. **Prieto-Alamo, M. J., J. Jurado, R. Gallardo-Madueno, F. Monje-Casas, A. Holmgren, and C. Pueyo.** 2000. Transcriptional regulation of glutaredoxin and thioredoxin pathways and related enzymes in response to oxidative stress. J. Biol. Chem. **275:**13398–13405.
37. **Qin, Z. S., L. A. McCue, W. Thompson, L. Mayerhofer, C. E. Lawrence, and J. S. Liu.** 2003. Identification of co-regulated genes through Bayesian clustering of predicted regulatory binding sites. Nat. Biotechnol. **21:**435–439.
38. **Rodionov, D. A., and M. S. Gelfand.** 2005. Identification of a bacterial regulatory system for ribonucleotide reductases by phylogenetic profiling. Trends Genet. **21:**385–389.
39. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
40. **Sekimizu, K., D. Bramhill, and A. Kornberg.** 1987. ATP activates dnaA

protein in initiating replication of plasmids bearing the origin of the *E. coli* chromosome. Cell **50:**259–265.
41. **Sinha, N. K., and D. P. Snustad.** 1972. Mechanism of inhibition of deoxyribonucleic acid synthesis in *Escherichia coli* by hydroxyurea. J. Bacteriol. **112:**1321–1324.
42. **Speck, C., C. Weigel, and W. Messer.** 1999. ATP- and ADP-dnaA protein, a molecular switch in gene regulation. EMBO J. **18:**6169–6176.
43. **Storz, G., and J. A. Imlay.** 1999. Oxidative stress. Curr. Opin. Microbiol. **2:**188–194.
44. **Sun, X., J. Harder, M. Krook, H. Jörnvall, B. M. Sjöberg, and P. Reichard.** 1993. A possible glycine radical in anaerobic ribonucleotide reductase from *Escherichia coli*: nucleotide sequence of the cloned *nrdD* gene. Proc. Natl. Acad. Sci. USA **90:**577–581.
45. **Torrents, E., P. Aloy, I. Gibert, and F. Rodriguez-Trelles.** 2002. Ribonucleotide reductases: divergent evolution of an ancient enzyme. J. Mol. Evol **55:**138–152.
46. **Torrents, E., G. Buist, A. Liu, R. Eliasson, J. Kok, I. Gibert, A. Gräslund, and P. Reichard.** 2000. The anaerobic (class III) ribonucleotide reductase from *Lactococcus lactis*. Catalytic properties and allosteric regulation of the pure enzyme system. J. Biol. Chem. **275:**2463–2471.
47. **Tuggle, C. K., and J. A. Fuchs.** 1990. Regulation of the operon encoding ribonucleotide reductase: role of the negative sites in *nrd* repression. J. Bacteriol. **172:**1711–1718.
48. **Vassinova, N., and D. Kozyrev.** 2000. A method for direct cloning of *fur*-regulated genes: identification of seven new *fur*-regulated loci in *Escherichia coli*. Microbiology **146:**3171–3182.