Two Proteins Mediate Class II Ribonucleotide Reductase Activity in Pseudomonas aeruginosa

EXPRESSION AND TRANSCRIPTIONAL ANALYSIS OF THE AEROBIC ENZYMES*

Received for publication, February 4, 2005, and in revised form, February 17, 2005 Published, JBC Papers in Press, February 17, 2005, DOI 10.1074/jbc.M501322200

Eduard Torrents‡§, Andrzej Poplawski‡§, and Britt-Marie Sjöberg¶

From the Department of Molecular Biology and Functional Genomics, Arrenhius Laboratories for Natural Sciences, Stockholm University, SE-10691 Stockholm, Sweden

The opportunistic human pathogen Pseudomonas aeruginosa is one of a few microorganisms that code for three different classes (I, II, and III) of the enzyme ribonucleotide reductase (RNR). Class II RNR of P. aeruginosa differs from all hitherto known class II enzymes by being encoded by two consecutive open reading frames denoted nrdJa and nrdJb and separated by 16 bp. Split nrdJ genes were also found in the few other γ-proteobacteria that code for a class II RNR. Interestingly, the two genes encoding the split nrdJ in P. aeruginosa were co-transcribed, and both proteins were expressed. Exponentially growing aerobic cultures were predominantly expressing the class I RNR (encoded by the nrdAB operon) compared with the class II RNR (encoded by the nrdDAB operon). Upon entry to stationary phase, the relative amount of nrdJa transcript increased about 6–7-fold concomitant with a 6-fold decrease in the relative amount of nrdA transcript. Hydroxyurea treatment known to knock out the activity of class I RNR caused strict growth inhibition of P. aeruginosa unless 5′-deoxyadenosylcobalamin, a cofactor specifically required for activity of class II RNRs, was added to the rich medium. Rescue of the hydroxyurea-treated cells in the presence of the vitamin B12 cofactor will grow in the absence of oxygen if nitrate is available as a respiratory electron acceptor (3). The opportunistic human pathogen P. aeruginosa is one of a few organisms to encode in its genome three different classes (Ia, II, and III) of the enzyme ribonucleotide reductase (RNR)1 (4). This essential enzyme catalyzes the reduction of ribonucleotides to the corresponding 2′-deoxyribonucleotides via a radical-dependent mechanism, thereby providing cells with the necessary building blocks for DNA synthesis (5). All known RNRs can be divided into three classes (I, II, and III) based on structural differences, metallocofactor requirements, and mechanisms used for radical generation (5–7). Class I RNRs, encoded by the nrdA and nrdB genes, are found in both prokaryotic and eukaryotic organisms. This class has a tetrameric (α2β2) structure consisting of two homodimeric proteins: R1 (α2), with the active site and allosteric sites, and R2 (β2), with a stable tyrosyl radical essential for catalysis and linked to a diiron-oxo center required for radical generation. The activity of class I RNR is restricted to aerobic conditions. Class II RNRs, encoded by the nrdJ gene, consist of a single polypeptide chain and are either monomeric (α) or homodimeric (α2). Class II RNRs use adenosylcobalamin (AdoCbl) in the radical generation process and operate both under aerobic and anaerobic conditions. This class has been found in archaea, eubacteria, and some lower eukaryotes (8). Class III RNRs, encoded by the nrdD gene, are homodimeric (α2) and carry a stable but oxygen-sensitive glycyl radical (9). They require a specific activase, encoded by the nrdG (β2) gene, that uses S-adenosylmethionine to generate the glycyl radical (10, 11). This class can only operate under anaerobic conditions and has been found in archaea and eubacteria (8).

Although there are significant differences between the three RNR classes, sequence alignments and in particular comparisons of their three-dimensional structures highlight striking similarities, including conservation of functional cysteines required for catalysis (12–14). All three RNR classes also share a sophisticated allosteric regulation mediated by different deoxy- and ribonucleotides, which guarantees an adequate and bal-

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1 The abbreviations used are: RNR, ribonucleotide reductase; AdoCbl, 5′-deoxyadenosylcobalamin; DTT, dithiothreitol; HU, hydroxyurea; ORF, open reading frame; RT, reverse transcription.
Splint Class II Ribonucleotide Reductase Protein

TABLE 1
Strains, plasmids, and oligonucleotides used in this study

| Strains          | Relevant features/sequence                                      | Origin or reference |
|------------------|-----------------------------------------------------------------|---------------------|
| E. coli          | recA1, endA1, hsdR, supE44, thi-1, gyrA96, relA1, ΔlacU169, deor Φ | Stratagene          |
| DH5α             | 80lacZM15                                                        |                     |
| S17-I            | recA1, thi-1, pro, hsdR, <RP4: 2-Tc: Mu:: KcsTn7, Tp#, Sm6, integrated into the chromosome |                     |
| P. aeruginosa    |                                                                  |                     |
| PAO1             | Type strain ATCC 6872                                            | Promega             |
| pGEM-T easy      | Broad-host-range expression system                               | This work           |
| pVLT31           | pVL371 vector carrying the nrdJa fragment                        | This work           |
| pETS131          | pVL371 vector carrying the nrdJa + nrdJb fragment                |                     |

**Oligonucleotides**

**Class I RNR (nrdA and nrdB)**
- F1-1280: 5'-GATCGTCCTCGTAAAGTGGTCA-3'
- F2-2853: 5'-GTCGACAGCTAATAAACCCTC-3'
- R1-3962: 5'-GGTAGGCGCGAGCTGAGA-3'
- R2-3567: 5'-TGGAGGGCTTCCGCTCCT-3'
- R3-1490: 5'-TCGAAGAGCGCTTTACGAA-3'

**Class II RNR (nrdJa and nrdJb)**
- F3-1548: 5'-TCCCTCGGAAGTATCCA-3'
- F4-2044: 5'-TCCGGATCAGCCGGAAGA-3'
- R4-2430: 5'-ACCATGTCGTTGGATGGTC-3'
- R5-1706: 5'-GGATAAGGTTGGGGCAATA-3'
- OP1-1TET-up: 5'-ATTGATATGGTCCCGTGAC-3'
- OP2-11-2w: 5'-AAACCTTCCAGACTTGGCCTGAGT-3'
- OP4-21-2w: 5'-AAACCTTCCAGACTTGGCCTGAGT-3'

16 S rDNA
- F5-1200: 5'-ACACGTGCTACAATGTTGCGCT-3'
- R6-130: 5'-TTCACCGAATGCATTGCTCA-3'

proC
- F6-447: 5'-CAGCCGGGCGAGTTGCTGCT-3'
- R7-615: 5'-GGTCAGGCGCGAGGCTGCT-3'

- F and R denote forward and reverse primers, respectively, and the number is the nucleotide sequence from the start codon.

Gene-specific primers used in RT-PCR and real time PCR were designed using the Primer Express ABI Prism program (Applied Biosystem), using the available information from the published *P. aeruginosa* genome sequence (24).

ancered supply of DNA precursors during DNA replication and repair. The essentiality of this enzyme makes it a good candidate for anticancer, antiviral, and antibacterial drug therapy (15–17). In *P. aeruginosa*, the investigation of the expression and physiological function of the three different classes of RNR is important from a biomedical and physiological point of view, and physiological function of the three different classes of RNR (15–17). In this study, we report that *P. aeruginosa* class II RNR (NrdJ) differs from all hitherto characterized RNRs in having a class II RNR (NrdJ) differs from all hitherto characterized RNRs in having a split nrdJ gene with two consecutive ORFs separated by 16 bp. Since class II RNR activity was earlier described in *P. aeruginosa* crude extracts (4), our discovery of the split nrdJ gene prompted further studies of functional aspects of class II RNR in *P. aeruginosa*. We show that class I and II RNRs were differentially expressed in aerobically growing *P. aeruginosa* and that class II is not a cryptic enzyme but requires both ORFs (NrdJa and NrdJb) for full activity and that it can support bacterial growth in the absence of class I RNR activity.

**MATERIALS AND METHODS**

**Bacterial Strains, Plasmids, and Growth Conditions**—All strains and plasmids used in this study are listed in Table I. Cells were cultured on Luria-Bertani (LB) medium at 37 °C. For solid media, Bacto agar (Difco) was added to a final concentration of 1.5%. When necessary, isopropyl-1-thio-β-d-galactopyranoside (1 mM) was added to the media, and antibiotics were added to the following final concentrations: for Escherichia coli, 10 μg/ml tetracycline and 50 μg/ml ampicillin; for *P. aeruginosa*, 50 μg/ml tetracycline.

**DNA and Protein Techniques**—Recombinant DNA manipulations and protein analyses were carried out according to published protocols (18). Genomic DNA from *P. aeruginosa* PAO1 was isolated using the DNeasy Tissue Kit (Qiagen) according to the manufacturer's specifications.

**RNA Extraction and Reverse Transcription-PCR**—For total RNA preparation, *P. aeruginosa* PAO1 cultures were mixed directly with RNase protect bacteria reagent (Qiagen). This reagent is designed for direct stabilization of bacterial RNA in *vitro*, and its protection from degradation during sample collection and storage (Qiagen). After mixing, samples were immediately frozen in liquid nitrogen and kept at −80 °C until RNA extraction, which took place less than a week after harvest. RNA was purified using an RNaseasy kit (Qiagen) in duplicates and pooled together after the extraction. An aliquot of the culture (at 1.4 × 10^8) equivalent to 10^9 cells was used for one RNA preparation. DNA contamination was removed from the RNA preparation by treatment with RNase-free DNase (Qiagen), followed by the RNase-free DNase digestion from Ambion according to the manufacturers' instructions. The amount of the RNA was determined from its 260-nm absorption using a Smart SpecTM spectrophotometer (Bio-Rad). RNA quality was estimated from the 260/280 ratio, which was equal to 1.9–2.0 in all preparations, and from electrophoresis on a 1% agarose gel.

For the RT step, 2 μg of *P. aeruginosa* total RNA was mixed with dNTPs (1 mM final concentration of each), and 10 μM of specific primers in a 20-μl reaction. The reaction mixture was heated at 65 °C for 5 min followed by quick transfer to ice. After the addition of buffer, 40 units of RNase inhibitor RnaseOUT, 15 units of Thermoscript reverse transcriptase (Invitrogen), and 5 mM DTT final concentration, 20 μl reactions were incubated at 60 °C for 50 min, followed by a 5-min inactivation step at 85 °C in a Robocycler 96 (Stratagene). In control samples (−RT in Fig. 1B), reverse transcriptase was omitted from the reactions, which were run in parallel in the Robocycler. To digest the RNA at the end of the RT reaction, 2 units of E. coli RNase H were added, and reactions were incubated at 37 °C for 20 min.

For the subsequent PCR amplification, 2 μl of the RT reaction (or negative control) was mixed with 0.2 μM each pair of primers, 0.2 mM of each dNTP, 5 μl of 10× PCR buffer and 1.5 units of High Expand Fidelity Taq polymerase (Roche Applied Science) in a total volume of 50 μl. For co-transcription studies of nrdAB, PCR mixtures were supplemented with MeSO to 0.4% final concentration. PCR amplifications were carried out under the following conditions: 94 °C for 180 s, followed by 35 cycles of 60 s at 94 °C, 60 s at either 49 °C (nrdAB studies) or 56 °C (nrdJa studies), and 90 s at 72 °C and completed by 420 s at
the XbaI and HindIII double-digested fragment containing the 2209-bp fragment was cloned into pGEM-T easy vector (Promega), and the region was cloned into the corresponding sites of pVLT31. A similar

In the subsequent real time PCR step, 100 ng of template with Taqman RT reagent kit (Applied Biosystems) at 48 °C for 50 min. For end point products of RT-PCR including both -RT and RT reactions were analyzed on 2% agarose gel containing ethidium bromide.

Quantitative RT-PCR Studies—To quantify class I and class II RNR transcripts from exponential to stationary growth phase as well as after HU treatment, several different protocols from Applied Biosystems, Qiagen and Invitrogen were tested. In the final protocol 0.5 μg of total P. aeruginosa RNA was reverse transcribed in the Robocycler in a 25-μl volume with Taqman RT reagent kit (Applied Biosystems) at 48 °C using random hexamers according to the manufacturer’s specifications. In the subsequent real time PCR step, 10 μl of a 10-fold diluted RT product corresponding to 20 ng of a total RNA was mixed with a 30-μl premix consisting of a ready to use qPCR Platinum® SYBR® Green qPCR SuperMix-UDG kit (Invitrogen), 0.8 μl of a 10-fold diluted RT product containing 0.5 μl of 50× concentrated ROX reference dye (Invitrogen), and 0.2 μM each of the selected specific primers. Negative controls (−RT) were run in parallel in each run. PCR amplifications were performed on an ABI Prism 7000 sequence detector (Applied Biosystems) in a 96-well optical reaction plate sealed with optical adhesive covers (Applied Biosystems). Before amplification, a uracil-DNA glycosylase decontamination step that prevents carryover contamination was performed. Each PCR product was amplified for 40 cycles of 10 s at 95 °C and 30 s at 62.5 °C with the ClustalX program (version 1.8.1) (22). For NrdJ proteins of the proteobacteria, we concatenated the protein sequences of the spilt NrdJ proteins (NrdJa + NrdJb). Neighbor joining was used to recover a protein distance matrix using ClustalX software. Default protein weight matrix (Blosum series) was used for alignments, and positions with gaps were excluded. The resultant tree was visualized with TreeView 1.6.6 (23). The following GenBank accession numbers were used for the NrdJa and NrdJb sequences: P. aeruginosa PAO1, NP_254194.1 and NP_25483.1; P. aeruginosa UCBPP-PA14, ZP_00140329.1 and ZP_00140328.2; A. vinelandii, ZP_00908261.1 and ZP_00098027.2; M. flagellatus, ZP_00173560.1 and ZP_00173561.2; P. profundum, YP_130341.2 and YP_130343.1; Magnetococcus sp., ZP_00290088.1 and ZP_00290089.1.

RESULTS

Genomic Organization of P. aeruginosa Class I and Class II Ribonucleotide Reductases—To study the genomic organization of class I and II RNR, we used the information from the complete P. aeruginosa PAO1 genome sequence (24). Class I RNR consisted of nrdA (PA1156) and nrdB (PA1155) genes encoding proteins of 963 amino acids and 415 amino acids linked together by a 263-bp intergenic region (Fig. 1A). Surprisingly, analysis of the class II RNR DNA region revealed a split of the nrdJ gene into two ORFs, a longer 2205-bp ORF (PA5497) and a shorter 690-bp ORF (PA5496), encoding proteins of 734 amino acids and 239 amino acids, which we name NrdJa and NrdJb, respectively (Fig. 1A). Sequencing of several PCR products covering the entire nrdJ intergenic region confirmed the split of the nrdJ gene into two parts by the presence of a UGA stop codon (cf. Fig. 6B).

To determine whether genes encoding class I or II RNR are co-transcribed, RNA from exponentially growing cells cultured in LB medium were isolated and reverse transcribed with a specific primer either to nrdB (R1-3962) or to nrdJb (R4-2430). Amplification of cDNA with primer pair F2–2853/R2–3567 for analyses of nrdA/nrdB or F4–2044/R4–2430 for nrdJ/nrdJb analyses (Fig. 1A) generated PCR products of the expected size (715 and 387 bp) and covered the entire intergenic regions of

![Fig. 1. Genomic organization and transcriptional analyses of class I and II RNR in P. aeruginosa. A, schematic representation of the genomic region of class I RNR genes (nrdA and nrdB) and class II RNR genes (nrdJa and nrdJb). B, coexpression of the class I and II RNR. Total RNA was reverse transcribed (RT) with gene-specific primers followed by PCR as described under “Materials and Methods.” +RT, positive reaction; −RT, negative controls for RT-PCR, where reverse transcriptase was omitted at the RT step to demonstrate the specificity of the reaction and the absence of DNA contaminations. End point products of RT-PCR including both +RT and −RT reactions were analyzed on 2% agarose gel containing ethidium bromide.](image-url)
Class II Enzyme Activity Requires Both NrdJa and NrdJb—Next we asked whether both NrdJa and NrdJb proteins are expressed. As shown by RT-PCR, nrdJa and nrdJb genes are co-transcribed, but the upstream part of nrdJb had no apparent consensus sequence for a ribosome-binding site as judged co-transcribed, but the upstream part of the protein (Fig. 2, corresponding to the expected molecular weight of the NrdJa ment (pETS131 plasmid) shows a strong band around 82 kDa pVLT31. Expression of the protein encoded in the fragment into the nrdJb genes are co-transcribed. Sequence analyses of several RT-PCR products from the nrdJ region confirmed the presence also at the mRNA level of the stop codon and the 16-S sequence separating the nrdJa and nrdJb genes.

Class II Enzyme Activity Requires Both NrdJa and NrdJb—Next we asked whether both NrdJa and NrdJb proteins are expressed. As shown by RT-PCR, nrdJa and nrdJb genes are co-transcribed, but the upstream part of nrdJb had no apparent consensus sequence for a ribosome-binding site as judged from comparison with the 3’ sequence of the 16 S rRNA (Gen-Bank™ accession number AB037545) from P. aeruginosa (25, 26). We therefore constructed two different expression derivatives by cloning either the nrdJa fragment or the entire nrdJb/nrdJb fragment into the Pseudomonas expression vector pVT31. Expression of the protein encoded in the nrdJa fragment (pETS131 plasmid) shows a strong band around 82 kDa corresponding to the expected molecular weight of the NrdJa protein (Fig. 2, lane 3). The plasmid carrying the whole nrdJ region (pETS133) shows expression of two major proteins with molecular masses of about 82 and 25 kDa, corresponding to the NrdJa and NrdJb proteins (Fig. 2, lane 4). These results confirm that both proteins are expressed in growing cells of P. aeruginosa. In addition, no band corresponding to the molecular mass of a fused NrdJa + NrdJb polypeptide was seen in any of our expression experiments.

Both NrdJa and NrdJb encode highly conserved RNR regions. NrdJa contains all the catalytic residues located at the active center of class II RNR including the three conserved cysteines (Cys-123, Cys-342, and Cys-353) needed for catalysis, and NrdJb carries the C-terminal cysteines that interact with the physiological reducing agents thioredoxin and glutaredoxin (12, 27) needed for the enzyme turnover cycle. Due to the split of these two important functions of the class II RNR we a priori expected an inactive enzyme, but a class II RNR activity was previously demonstrated in a crude extract of P. aeruginosa cells (4). We therefore used the plasmid constructs to test whether both proteins were needed for a functional class II RNR or whether the NrdJa protein was enzymatically active by itself when supplied with an artificial hydrogen donor like DTT. To avoid measuring intrinsic class I RNR activity, we used dATP as allosteric effector (negative effector of class I and positive effector of class II RNR) and included the class I-specific inhibitor HU in some assays. As shown in Table II, a crude extract of P. aeruginosa carrying the pVLT31 vector without inserts has almost no activity without the class I-specific cofactor AdoCbl, and it is only after the addition of AdoCbl that we observe a class II RNR activity of about 0.068 units/mg protein, which corresponds to the previously found activity in crude extracts of P. aeruginosa (4). When NrdJa is expressed from pETS131, the specific activity increases somewhat to 0.169 units/mg when AdoCbl is present in the assay. A dramatic increase is obtained when both proteins (NrdJa and NrdJb) are expressed from plasmid pETS133, in which case the class II activity is 10-fold higher than the class II background activity in crude extract. In addition, the enzyme activity in the pETS133 extract is completely AdoCbl-dependent, because no activity is present without this cofactor.

Expression of Class I and II RNR as a Function of Growth Phase—Next, we investigated the differential expression of class I (nrdA) and class II (nrdJa) RNR of P. aeruginosa during different growth phases by quantitative real time PCR. As shown in Fig. 3, expression of both nrd genes clearly are dependent on the culture growth conditions but with opposite expression pattern. Class Ia RNR (nrdA) reached its highest expression at the beginning of the exponential phase and decreased dramatically at the end of the exponential phase, where it was 6–7-fold lower compared with exponential phase. On the other hand, class II RNR (nrdJa) transcription followed an inverted pattern and was increased at the end of the exponential growth phase, and in stationary phase the transcription was 6-fold higher than in exponential phase. Notably, the overall level of expression differs greatly between the two genes. Whereas the highest expression of nrdA reached a level of about 140 mRNA copies per 10^4 S rRNA copies, the highest level of the nrdJ was about 7 mRNA copies per 10^4 S rRNA copies.

Adenosylcobalamin Required for Growth of P. aeruginosa in the Presence of HU—Although both class I and II genes are transcribed simultaneously (this study) and both enzymes are active under normal growth conditions (4), we still do not know if both classes are important for survival of P. aeruginosa. We therefore asked whether the class II RNR could support growth in the absence of class I activity. As before, we used the radical scavenger HU to knock out class I activity and added the co-factor AdoCbl to promote class II activity. After 16 h of incubation on solid medium at 37 °C, a first inspection of the plates revealed that AdoCbl did not have any inhibitory effect on P. aeruginosa viability compared with the untreated control culture (Fig. 4). Plates supplemented with HU, however, showed almost no growth due to the inhibition of class I RNR, suggesting that class I RNR supports growth of P. aeruginosa on defined media. Interestingly, plates containing both HU and AdoCbl showed overnight growth for both undiluted and 10^-1 diluted culture (not shown). Therefore, incubation of the plates was continued for an additional 24 h before final growth results were recorded. The inhibitory effect of hydroxyurea on P. aeruginosa was still evident, even after prolonged incubation and survival was <10^-5 compared with untreated control plates (Fig. 4). Cells receiving 1 mg/ml AdoCbl in the presence of HU had a striking increase of viability after prolonged incubation and showed substantial growth in the 10^-2-fold diluted culture and some growth even in the 10^-4-fold diluted culture (Fig. 4). The same experiment using cyanocobalamin (which does not function as a co-factor for class II RNR) instead of AdoCbl gave similar results (data not shown), corroborating experimentally that P. aeruginosa can import and metabolize cyanocobalamin to the required type of cobalamin (28).
Hydroxyurea Induces Expression of Both Class I and II RNR Genes under Aerobic Conditions—*P. aeruginosa* was grown in LB medium, and in early log phase part of the culture was supplemented with HU, part of the culture was supplemented with HU plus AdoCbl, and incubation was continued. Total RNA was extracted from aliquots withdrawn at different times, and the levels of *nrdA* and *nrdJ* expression were quantified by real time PCR. Due to recurring variability in the 16 S rRNA initially used for data normalization in these experiments, we instead used the housekeeping *proC* gene for data normalization. Cells were grown to an A_{600} of 0.12 and then shifted to inducing conditions. Curve shows -fold changes in *nrdA* (circles) and *nrdJ* (squares) expression from several experiments after exposure of *P. aeruginosa* to either 5 mM HU (○, □) or 5 mM HU/1 μg/ml AdoCbl (●, ■) relative to those in the untreated culture (see “Materials and Methods”).

**DISCUSSION**

*P. aeruginosa, Bacteroides fragilis, Clostridium acetobutylicum*, and a few proteobacteria are distinctive in that they contain genes for three different classes of RNR (Ia, II, and III) in their genomes. *P. aeruginosa* is also distinct from most other class II-containing prokaryotes in having a genomic class II region that is split into two consecutive genes (*nrdJa* and *nrdJb*). In the present study, we have focused on the two *P. aeruginosa* RNR classes that work under aerobic conditions (Ia and II) and determined the physiologically active form of the class II enzyme and the roles of class I and II RNR during aerobic growth of *P. aeruginosa*.

At first glance, the class II RNR region in *P. aeruginosa* looks like a pseudogene. Conserved and known vital parts of the class II RNR family are split in the *P. aeruginosa* genome by a TGA stop codon, and the two resulting open reading frames (*NrdJa* and *NrdJb*) are separated by a 16-bp spacer. However, an earlier report had identified class II RNR enzyme activity in crude extracts of *P. aeruginosa* (4). In this study, three approaches were used to corroborate this unexpected split of the *nrdJ* gene: DNA sequence analyses, sequence analyses of RT-PCR products resulting from expression analyses, and protein expression analyses. DNA sequencing of several stocks of PAO1 confirmed the presence of the stop codon in the genomic *nrdJ* region. Our RT-PCR analyses clearly showed that both genes (*nrdJa* and *NrdJb*) are linked and transcribed as a single mRNA. Sequencing of the cDNA from the RT-PCRs also confirmed the presence of the stop codon in the genomic *nrdJ* region. The amounts of *nrdA* and *nrdJ* transcripts in both control and treated samples were calculated from cDNA calibration curves, followed by normalization based on 16 S rRNA levels (see “Materials and Methods”).

**FIG. 4.** The effect of hydroxyurea and adenosylcobalamin on viability of *P. aeruginosa*. Drops of 10 μl from 0, 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} dilutions of an exponentially growing culture (A_{600} = 0.4) were spotted on solid medium containing LB without additions or with 1 μg/ml AdoCbl or 5 mM HU or with both 5 mM HU and 1 μg/ml AdoCbl. Plates were incubated at 37 °C for 40 h and were photographed using a Nikon Coolpix 4300 camera. Digitized images were prepared using Adobe Photoshop software.

**FIG. 5.** Expression of *P. aeruginosa* class I and II RNRs after the addition of HU or HUAdoCbl. The amounts of *nrdA* and *nrdJ* transcripts in both control and treated samples were calculated from cDNA calibration curves, followed by normalization on the basis of *proC* endogenous control gene expression. Cells were grown to an A_{600} of 0.12 and then shifted to inducing conditions. Curve shows -fold changes in *nrdA* (circles) and *nrdJ* (squares) expression from several experiments after exposure of *P. aeruginosa* to either 5 mM HU (○, □) or 5 mM HU/1 μg/ml AdoCbl (●, ■) relative to those in the untreated culture (see “Materials and Methods”).
passing the allosteric specificity site and the active site and
that NrdJb contains the C-terminal cysteine cluster that inter-
acts with the physiological reducing system (thioredoxin and
glutaredoxin system) (12, 27). As expected for a class II RNR (8,
30), no consensus sequence for an allosteric activity site was
present in the N-terminal part of NrdJa. It was earlier reported
that a Thermotoga maritima engineered class II RNR lacking
183 amino acid residues at the C-terminal region is enzymat-
ically active in the presence of DTT (30). Here we show that the
P. aeruginosa NrdJa protein totally lacks enzyme activity with
DTT even in the presence of wild type crude extract, which also
contains all the physiological reducing agents. The P. aerugi-
nosa class II RNR only shows enzyme activity when both NrdJa
and NrdJb proteins are present. This is the first demonstration
of a class II RNR that requires a two-component system for
enzyme activity. Class Ia/Ib RNRs require both the active site-
containing NrdA/NrdE and the radical-containing NrdB/NrdF
for a complete functional enzyme. Class III RNRs require the
NrdG protein to generate the glycyl radical on the catalytic
subunit (NrdD). However, once NrdD has acquired the glycyl
radical, this component alone can perform multiple turnovers
(31), so the enzymatically active form of class III RNR consists
only of NrdD.

Class II RNRs require the vitamin B12 coenzyme AdoCbl for
activity. The recently solved three-dimensional structures for
the class II enzymes from Lactobacillus leichmannii and T.
maritima indicate that the cofactor binds close to the active site
region (32–34). AdoCbl binding may involve a conserved argi-
nine in the N-terminal part of the polypeptides as well as
regions in the more C-terminal part of the polypeptides. How-
ever, these structures do not allow a detailed description of the
AdoCbl binding, and the T. leichmannii and T. maritima differ
substantially in amino acid sequences from the P. aeruginosa
class II sequence. At this point, we can merely speculate that
the function of P. aeruginosa NrdJb might be to sequester and
present AdoCbl to NrdJa or that a binding pocket for AdoCbl is
formed until NrdJb has bound to NrdJa. In addition, the
very C-terminal part of NrdJb is plausibly required for enzyme
turnover in vivo.

Interestingly, BLAST analyses revealed that a split nrdJ gene
was not unique to class II RNR in P. aeruginosa PA01, since this
feature was also found in the P. aeruginosa strain UCBPP-PA14
and in Azotobacter vinelandii, Magnetotococcus sp., Methylobac-
tillus flagellatus, and Photobacterium profundum. The phylogenetic
tree in Fig. 6A shows a prediction of the relationship between
the known NrdJ proteins from Gram-negative proteobacteria.
The most salient feature is the clear separation of the split
NrdJ sequences, which basically correspond to the proteobacteria
γ-subdivision, are marked with a dark gray background, and sequences belonging to the α, β, γ-subdivision of the proteobacteria are marked with a light gray background. The tree was generated with 1000 bootstrap trials, and the bootstrap values are indicated at the nodes. B, alignment of the intergenic regions of the split nrdJ genes. The stop codons of the nrdJa genes and the start codons of the nrdJb genes are indicated by white letters on a black background. Relevant nucleotide sequences were from the following GenBank™ accession numbers: P. aeruginosa PA01, NC_002516; P. aeruginosa UCBPP PA14, NZ_AABQ00000000; A. vinelandii, NZ_AAAU0200008; M. flagellatus, NZ_AADX01000002.1; P. profundum, NC_006370.1; Magnetococcus sp., NZ_AAN02000010.1.
to note that class II RNR is uncommon among the \(\gamma\)-proteobacteria where mainly classes Ia, Ib and III are found (8), and the only class II RNR found in this subdivision is the split variant. The data suggest that the split of the \(nrdJ\) gene has occurred once, because all split genes are clustered together (Fig. 6A). The amino acid sequence identities are quite high (52–79%) among the split NrdJs compared with most other protobacterial NrdJs.

Further analyses at the DNA level of the six split \(nrdJ\) sequences revealed that also the position of the stop codon is well conserved among all of these sequences (Fig. 6B). The intergenic regions, on the other hand, differ extensively in length (from 16 to 108 bp) and in sequence. A distinct nucleotide/amino acid sequence similarity between the \(nrdJ\) genes is not obvious until about 5–12 codons downstream of their ATG start codons (data not shown).

The fact that the split class II RNR is enzymatically active is a strong indication that it is essential to \(P. aeruginosa\). A major question that follows is under what growth conditions the split \(nrdJ\) gene is expressed and whether it can support growth of \(P. aeruginosa\). RT-PCR analyses revealed that both the \(nrdAB\) and the \(nrdJab\) operon are expressed in aerobically growing \(P. aeruginosa\). More accurate real time PCR quantification of the mRNA for each class (I and II) showed that their expression patterns are growth cycle-dependent (Fig. 3), as is generally expected for expression of \(nrd\) genes (35, 36). Only class I is highly expressed at the beginning of the exponential growth, clearly indicating that class I RNR supports the standard laboratory aerobic growth of \(P. aeruginosa\). Significant class II expression is not observed until the bacteria are approaching stationary phase, when \(nrdJ\) expression was increased about 6-fold. At the same time, class I transcription is drastically down-regulated.

What might the function of the two-component NrdJ system be? It is believed that only low amounts of deoxyribonucleotides are needed during stationary phase growth, primarily for DNA repair rather than for DNA replication. Likewise, the radical scavenger HU, which is a specific inhibitor of class I RNR, is also believed to indirectly induce DNA repair processes via the SOS response pathway (37, 38). Notably, the expression of \(nrdJ\) was highly induced in stationary phase and after HU treatment in our experiments, possibly suggesting that the function of the split \(nrdJ\) gene product is primarily for DNA repair. In the same vein, class II RNR could only support growth if we supplemented the defined laboratory media with the class II-specific cofactor AdoCbl, suggesting that their expression patterns are growth cycle-dependent and that expression of class II RNR is not under vitamin B12 control, as suggested for several other RNRs (28, 39, 40), because all split genes are clustered together (Fig. 6).

Based on our combined genetic and biochemical results, we propose that class I RNR supports aerobic DNA replication and that the unique two-component class II RNR is used for repair and/or possibly replication at low oxygen tension. We are currently investigating the physiological importance of the split \(nrdJ\) gene in \(P. aeruginosa\) during infection of eukaryotic model organisms.

Acknowledgments—We are grateful to Prof. Victor de Lorenzo for the generous gift of some plasmids and strains used in this study and to MariAnn Westman for excellent technical help.

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