Corynebacterium ammoniagenes class Iβ ribonucleotide reductase: transcriptional regulation of an atypical genomic organization in the nrd cluster

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Ribonucleotide reductases (RNRs) are a family of complex enzymes that play an essential role in all organisms because they catalyse de novo synthesis of deoxyribonucleotides required for DNA replication and repair. Three different classes of RNR have been described according to their metal cofactors and organic radicals. Class I β RNR is encoded in four different genes (nrdH, nrdl, nrdE and nrdF) organized in an operon. The authors previously cloned and sequenced the genes encoding the active class Iβ RNR of Corynebacterium ammoniagenes and showed that these genes are clustered in an atypical nrdF region, which differs from that of other known class Iβ enzymes because of an intergenic sequence (1171 bp) present between nrdE and nrdF. This study investigated the transcriptional organization and regulation of this nrd region by RT-PCR. Three different and independent mRNA were found (nrdHIE, nrdF and an ORF present in the intergenic region), each one being transcribed from its own promoter and being essential for normal growth. The ratio of nrdF to nrdHIE mRNA was 9:1, as determined by competitive RT-PCR; the expression of both nrdHIE and nrdF was found to be dependent on the culture growth phase, and was induced in the presence of hydroxyurea, manganese and hydrogen peroxide. This is believed to be the first direct evidence for a manganese-dependent transcriptional regulation of nrd genes. These results suggest a common and coordinated regulation of the different nrd genes, despite their being transcribed from independent promoters.

INTRODUCTION

Ribonucleotide reductases (RNRs) catalyse the reduction of ribonucleotides to their corresponding 2′-deoxyribonucleotides and therefore play an essential role because they synthesize the monomers necessary for DNA polymerization. There are three classes of RNR, with different primary structures, subunits, cofactor requirements and regulation, but they have in common the use of an organic radical to initiate catalysis through free-radical chemistry and allosteric regulation (Jordan & Reichard, 1998).

Class I (αβγ) RNRs contain a stable tyrosyl radical and an oxygen-linked diferric centre which is required for radical generation. Such a process requires oxygen, and therefore this class functions only under aerobic conditions. Class I has been divided into classes Iα (NrdAB) and Iβ (NrdEF) (Jordan et al., 1996b). Class II enzymes (α2 or z) require S-adenosylcobalamine as a radical generator and do not depend on oxygen. Members of class III (αz) contain a free radical located on a glycy1 residue, plus an iron–sulfur centre that catalyses the reduction of S-adenosylmethionine to generate this radical. These enzymes only work under strict anaerobic conditions. In all classes, the z subunit binds substrates and allosteric effectors and is the catalytic part of the enzyme. Class Iα RNR is found in all types of eukaryotic cells and viruses, archaea and some eubacteria; classes II and III are found in archaea and eubacteria, while class Iβ is only found in eubacteria.

Class Iβ RNR was discovered in Salmonella typhimurium, without any apparent function (Jordan et al., 1996a) because class Iα was also present and it was shown to be the active class under standard laboratory growth conditions. Recent studies demonstrated a potential role for the class Iβ enzyme under some special growth conditions (Masalha et al., 2001; Monje-Casa et al., 2001). A distinguishing feature of all known class Iβ operons is that they code for a

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Abbreviations: DIP, 2,2′-dipyridyl; RNR, ribonucleotide reductase.
small protein, Nrld, of unknown function but seemingly essential for cell growth (I. Sala, unpublished data), and often for a specific external electron donor (Nrhd1). Class Ib nrd genes are typically arranged in an operonic fashion following the pattern nrdH–nrdI–nrdE–nrdF. Because of their importance, RNRs are tightly regulated by both cell cycle and environmental cues to provide a sufficient pool of balanced dNTPs for DNA replication and repair. This fine regulation is achieved by at least two different regulatory mechanisms: allosteric control of the enzyme and transcriptional regulation of the genes. While a lot of research has been carried out concerning the allosteric regulation of the enzyme (Jordan & Reichard, 1998), very little is known about the transcriptional mechanisms that regulate expression of an active class Ib nrd genes.

Oka et al. (1968) showed that manganese limitation produced unbalanced growth and DNA synthesis arrest in coryneform bacteria by inhibiting the synthesis of DNA precursors. When manganese was added to the medium, DNA synthesis and cell growth were rapidly restored to the levels of non-manganese-starved cells. It was suggested that the target for this manganese starvation was the RNR (Schimpff-Weiland et al., 1981) and this observation led to the classification of this enzyme into a new class of RNR, class IV (Stubbe & van der Donk, 1995). Some years ago, we cloned and sequenced the genes that code for the active RNR of C. ammoniagenes (Fieschi et al., 1998) and we proved that iron, rather than manganese, was the metal involved in the generation of the tyrosyl radical (Huque et al., 2000). Gene sequencing, and enzyme, radical and metal cofactor characterization, corroborated the view that C. ammoniagenes RNR was a true class Ib enzyme. The class Ib RNR genes of C. ammoniagenes are arranged in an atypical nrd organization when compared to other nrdHIEF genes because an intergenic region (containing an ORF in the opposite strand) is present between nrdE and nrdF. Although C. ammoniagenes is not a textbook bacterium and only a few molecular tools for its genetic manipulation are available, in this study we have focused on analysing the transcriptional organization and genetic regulation of its active class Ib RNR enzyme, in an attempt to elucidate the role that manganese or other environmental factors might play in its transcriptional regulation.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** All strains and plasmids used in this study are listed in Table 1. Escherichia coli and C. ammoniagenes strains were routinely grown in Luria–Bertani broth (LB) at 37°C. Antibiotics and chromogenic substrates, when required, were included in the culture media or plates at the following concentrations: ampicillin 50 μg ml⁻¹, kanamycin 50 μg ml⁻¹, chloramphenicol 30 μg ml⁻¹, X-Gal 30 μg ml⁻¹ for E. coli; and kanamycin 50 μg ml⁻¹ and chloramphenicol 8 μg ml⁻¹ for C. ammoniagenes. In order to create iron-limiting growth conditions, the iron chelator 2,2'-dipyridyl (DIP) was added at 150 μM to LB liquid cultures.

**General recombinant DNA techniques and sequence analysis.** Plasmid DNA preparation and transformation, restriction enzyme digestions and ligations were done by standard techniques (Sambrook et al., 1989). DNA fragments were purified from agarose gels by melting the band in 6 M NaI at 50°C and using the Wizard DNA clean-up kit (Promega). General recombinant DNA techniques and sequence analysis were done by standard protocols (Sambrook et al., 1989) and the plasmid DNA was analysed by agarose gel electrophoresis.

**Plasmid DNA preparation and transformation.** Plasmid DNA was prepared from DH5α, DH10B or C. ammoniagenes strains by the alkaline lysis method (Hoeffer, 1983). Plasmid DNA was purified from agarose gels by melting the band in 6 M NaI at 50°C and using the Wizard DNA clean-up kit (Promega). General recombinant DNA techniques and sequence analysis were done by standard protocols (Sambrook et al., 1989) and the plasmid DNA was analysed by agarose gel electrophoresis.

**Table 1. Strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or characteristics</th>
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<td><strong>E. coli</strong></td>
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</tr>
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<td>ATCC 12435</td>
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<td>pK18mob</td>
<td>E. coli cloning and sequencing vector, Km′</td>
<td>Schäfer et al. (1994)</td>
</tr>
<tr>
<td>pCM502</td>
<td>Promoter-probe shuttle vector carrying the promoterless β-galactosidase gene, Cm′</td>
<td>Schmitt (1997)</td>
</tr>
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<td>pUA728</td>
<td>SacI C. ammoniagenes genomic fragment containing the entire nrdEF region</td>
<td>Fieschi et al. (1998)</td>
</tr>
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<td>pETS115</td>
<td>pBSK carrying the nrdF promoter</td>
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</tr>
<tr>
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</tr>
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<td>pETS126</td>
<td>pK18mob carrying a 961 bp DNA fragment of the sugar transporter gene</td>
<td>This study</td>
</tr>
<tr>
<td>pETS127</td>
<td>pK18mob carrying a 455 bp DNA fragment of nrdHIEF</td>
<td>This study</td>
</tr>
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DNA Clean-up system (Promega). DNA sequence determination was carried out using the BigDye Terminator kit and an ABI prism 310 sequencer (Applied Biosystems).

**C. ammoniagenes electrotransformation procedure.** *C. ammoniagenes* was grown in 10 ml LB broth at 30 °C until the culture reached an OD550 of 0.4–0.6. The cells were kept on ice for 5 min and harvested by centrifugation in a polypropylene tube at 5000 r.p.m. at 4 °C for 10 min. The pellet was resuspended in 1 ml cold sterile distilled water and transferred into an Eppendorf tube. The cells were then washed three more times with distilled water in a microcentrifuge. Any remaining supernatant was removed with sterile tips before resuspending the cells in 1 ml ice-cold 10% (v/v) glycerol. Finally, the cells were resuspended in 80 μl ice-cold 10% glycerol, and kept on ice prior to electroporation. For electroporation, 40 μl fresh electroporant competent cells were mixed with plasmid DNA (1 μg) in a cold sterile electroporation cuvette (2 mm electrode gap, Biotechnologies and Experimental Research, BTX) and pulsed immediately with a BTX Electro Cell Manipulator 600. The cell manipulator was usually set at a voltage of 2-5 kV, parallel resistor 326 Ω. The cell solution was resuspended in 1 ml BHI (Oxoid) and then withdrawn immediately using a sterile Pasteur pipette and incubated at 37 °C for 3 h before plating.

**Southern blot analysis.** Chromosomal DNA from *C. ammoniagenes* was isolated as described by Fieschi et al. (1998). DNA was digested, separated by agarose (0.7% g/l) gel electrophoresis and vacuum-transferred to nitrocellulose membranes. Specific *nrd* probes were obtained from different sources: *nrdD* from *Lactococcus lactis* (Torrents et al., 2000a), *nrdF* from *Corynebacterium nephridii* (M. Karlsson, unpublished data), *nrdE*, *nrdI* and *nrdL* from *C. ammoniagenes* (Fieschi et al., 1998), *nrdA*, *nrdB* and *nrdD* from *Pseudomonas aeruginosa* (Jordan et al., 1999) and *nrdA*, *nrdD* and *nrdF* from *S. typhimurium* (Torrents et al., 2000b). Probes were isolated by PCR and labelled with dUTP-digoxigenin. High- and low-stringency conditions were used for hybridization and washing. Southern blots were subjected to chemiluminiscence detection, as previously described (Torrents et al., 2000b).

**Construction and determination of β-galactosidase fusions.** The *C. glutamicum* promoter-probe pCM502 vector (Schmitt, 1997) was used to construct a transcriptional fusion of the *nrdHIE* and ORF–*nrdF* promoter regions to the lacZ gene. A 698 bp fragment containing the ORF and *nrdD* promoter regions was obtained by PCR amplification using primers 10 and 8 (Table 2) and cloned into the pGEM-T easy vector. This fragment was further removed by EcoRI digestion and transferred into a pBSK(+) vector, generating pETS115 and pETS116, according to the orientation of the insert, as checked by restriction mapping. A BamHI–SalI fragment from each of these constructs was ligated into pCM502, generating pETS121 and pETS122, which contained, respectively, the properly oriented *nrdF* and ORF promoters. For the construction of the *nrdHIE–lacZ* fusion, a 1716 bp EcoRV fragment encompassing 1624 bp of the region upstream from the start codon of *nrdD*, derived from pUA278, was cloned into pBSK(+), generating pETS118. This plasmid was digested with BamHI and SalI and was cloned into pCM502, generating pETS123. The plasmids containing the fusions were introduced into *C. ammoniagenes* by electroporation and were selected for their chloramphenicol resistance. β-Galactosidase specific activities were measured in cell extract and were expressed in Miller units (Miller, 1972) but corrected against protein concentration instead of the optical density of the culture. All enzyme assay data are the mean values from at least three independent experiments. Samples were taken at the times specified in each experiment.

**Western immunoblot analysis.** Cells from a mid-exponential-phase culture (OD550 0.4–0.6, 1·5 ml culture) of *C. ammoniagenes* were collected by centrifugation and resuspended in 10 μl 10 mM Tris/HCl pH 7.5 and 10 mg lysozyme ml⁻¹ at 37 °C. After 15 min, 50 μl cracking buffer (0·5 M Tris/HCl pH 8, 10% SDS, 5%, (v/v), 2-mercaptoethanol and 0·1% bromophenol blue) was added and boiled for 10 min. After centrifugation, samples were resolved by 7·5% SDS-PAGE and electrotransferred onto nitrocellulose membranes (Bio-Rad) using a Mini-PROTEIN II system (Bio-Rad), according to the manufacturer’s protocol. Membranes were blocked in a solution of 3% (w/v) dry milk in PBS overnight at 4 °C. Monoclonal anti-β-galactosidase was used at 100 ng ml⁻¹ and anti-mouse horseradish peroxidase conjugate at a dilution of 1:3000 (Roche Diagnostics). The antibody–antigen complex was detected using the BM Chemiluminescence Blotting Substrate (POD) kit from Roche Diagnostics, according to the manufacturer’s protocol.

**Gene disruption.** The method described by Schwarzer & Pühler (1991) was used to perform gene disruption. Briefly, an internal gene fragment from *nrdI* and *nrdD* was amplified by PCR using wild-type chromosomal DNA as a template and primers 2 and 3 for *nrdI* and 7 and 9 for *nrdD*. As a positive control we used a 961 bp BamHI fragment of the pUA278 plasmid corresponding to a sugar transport protein (STP) (GenBank AJ504720). The resulting 455 bp, 719 bp and 961 bp fragments from the *nrdI*, *nrdD* and STP, respectively, were ligated into plasmid pEH18mob, which is non-replicative in *C. ammoniagenes*. These ligations generated plasmids pETS127, pETS125 and pETS126, which were introduced into *C. ammoniagenes* by electroporation and plated on Km-LB plates. Kanamycin-resistant cells corresponded to the transconjugants carrying the integrated plasmids. To confirm the insertions, Southern hybridization was carried out as described above.

**RT-PCR.** Total cellular RNA was extracted from mid-exponential-phase (OD550 0.5) *C. ammoniagenes* cultures (200 μl) grown at 37 °C, by using the High Pure RNA Isolation Kit from Roche Diagnostics, according to the manufacturer’s protocol. Equal amounts of total RNA (3 μg) were used for cDNA synthesis. The reaction mixture in a total of 21 μl contained 4 μl 5× RT-buffer (Promega), 2 μl dNTPs mix (2 mM each), and 25 pmol primer RTlow. After incubation at 65 °C for 5 min, primer annealing was performed at room temperature for 5 min, and RNAs inhibitor (4 U) (Roche Diagnostics) and AMV reverse transcriptase (10 U) were added. The reaction was incubated at 42 °C for 45 min. The cDNA was diluted 1:2 before further use.

<table>
<thead>
<tr>
<th>Primer no.</th>
<th>Sequence (5′–3′)</th>
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<tr>
<td>2</td>
<td>GTTCTGTGCGTGATACCTC</td>
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<tr>
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<td>TTTTAGCTGCGCTTGTCTTTT</td>
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<tr>
<td>4</td>
<td>GTGACTGCAACAAATTG</td>
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<tr>
<td>5</td>
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<td>6</td>
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<tr>
<td>18</td>
<td>AAGGGCGATGATGATATTCG</td>
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</table>

**Table 2. Primer sequences used in this work**
(Promega) were added to perform the cDNA synthesis at 48 °C for 60 min; the reaction was terminated by incubation at 94 °C for 5 min. cDNA aliquots of 2 μl were directly used for PCR. cDNA amplification reaction in 50 μl total volume was performed in a solution containing 50 pmol of each primer, dNTPs mix (0-2 mM each), 5 μl 10 × PCR buffer and 1-5 U High expand Taq polymerase (Roche Diagnostics). Amplifications were carried out in a PTC-150 MiniCycler (MJ Research) under the following conditions: denaturation at 94 °C for 3 min followed by 30 cycles at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min and a final polymerization step at 72 °C for 7 min. Samples (15 μl) of each PCR reaction were analysed by 2 % Nusieve-GTG-agarose (FMC Bioproducts) gel electrophoresis. Before RT-PCR, all samples were proven to be free of contaminating DNA by performing control PCRs without a preceding RT step. Buffers, cycling conditions and the amount of template were the same as for RT-PCRs.

**Competitive RT-PCR.** A deletion in length but no difference in primer sequence could distinguish the native nrdE and nrdF genes from the competitor crrdE and crrdF genes. The nrdE deletion was carried out as an EcoRI digestion, generating a 220 bp deletion. To generate the nrdF deletion, we used a combination of primer pairs 7+16 and 15+9 (Table 2). Once amplified, both fragments were digested with EcoRI and were ligated. The deleted nrdF fragment was generated by PCR amplification (see above for PCR protocol) using the previous ligation. This 543 bp fragment was further cloned into the pGEM-T easy vector.

The RT and PCR conditions used were as described above for RT-PCR, with the addition of a constant amount (500 ng) of competitor at dilutions 10⁻⁶ and 10⁻⁷ for nrdHIE or 10⁻⁵ and 10⁻⁶ for nrdF. Levels of each product were corrected for size, to compensate ethidium bromide incorporation during the gel electrophoresis. Afterwards, the ratio of amplified target to competitor product was determined for the nrdE and nrdF genes. Variations in the ratios indicate the relative differences in initial mRNA levels between the samples and are calculated according to Jordan et al. (1996a).

**Image and data analysis.** Images of each gel were captured with a Gel Doc 2000 station and Quantity One software package (Bio-Rad). For quantitative RT-PCR, individual band intensities were quantified and the localized background was subtracted from the computer-determined value for each band.

**RESULTS**

**Transcriptional analysis of the nrd region**

Initial attempts to analyse the transcriptional structure of the identified nrd genes were performed by Northern blotting. RNA was prepared and hybridized with denatured digoxigenin-labelled DNA probes against nrdE and nrdF as described by Jordan et al. (1996a). This method was not sensitive enough to detect the nrd mRNA, indicating low transcriptional levels (data not shown). To circumvent these problems, RT-PCR assays were carried out. The locations of the primer pairs used for the detection of nrdH, nrdl, nrdE, nrdF and the ORF are shown in Fig. 1(a) and the primer sequences are listed in Table 2.

Total RNA was reverse-transcribed with primer 5 located at 849 bp within nrdE. This product was amplified using

![Fig. 1. Genetic organization of the 7.1 kb nrd cluster in C. ammoniagenes and gene linkage. (a) Open arrows, drawn to scale, represent the locations and orientations of the nrd genes. The white open arrow indicates the flanking sugar transporter protein (STP) gene. In this region, genes for a NrdH-redoxin (nrdH), the Nrdl protein (nrdl), the large (NrdE) and the small (NrdF) RNR subunits (encoded by nrdE and nrdF) can be found. Small solid arrows show primers used for RT and PCR reactions (Table 2). Note that length and position are not drawn to scale. Possible promoters are marked P→. Putative mRNA transcripts are indicated by arrows labelled A, B and C. (b) Non quantitative RT-PCR in the nrd region. The linkage strategy is outlined in (a) and the primer sets used (see Table 2) are indicated at the top. RT-PCR was conducted as described in Methods without the addition of any competitor. M, molecular size markers.](image-url)
promoters 3 and 1 and primers 1 and 5. The reaction amplified specific products of 789 bp and 1716 bp that corresponded in size to a product amplified from chromosomal DNA (Fig. 1b, lanes HI and HIE), indicating that nrdH, nrdI and nrdE were linked and present on the same transcript (Fig. 1a, arrow A). The nrdF transcript was detected independently from the previous messenger using primer 9 to generate first-strand cDNA, and primers 7 and 9 in the PCR amplification (Fig. 1b, lane F). However, all attempts to detect a single transcript spanning the whole region failed. We used primers 8, 12 and 9 in the RT reaction and primers 9 and 6, 8 and 6, and 12 and 6 in the PCR, but no amplification fragments were detected. These results indicated two independent mRNAs for nrdHIE and nrdF (Fig. 1a, arrows A and B).

Interestingly, an mRNA transcript was found in the intergenic region in the opposite direction corresponding to an ORF predicted in the DNA sequence. Using primer 11 in the RT reaction and primers 11 and 12 in the PCR reaction, we detected a transcript within the ORF region (Fig. 1b, lane ORF) which corresponds to transcript C in Fig. 1(a).

**Promoter expression analysis with lacZ fusions**

In order to confirm the presence of different promoter regions associated with the previously detected transcripts (Fig. 1a), sequences upstream of nrdH, nrdF and the ORF were cloned into the promoter-probe vector pCM502 to create transcriptional fusions with the lacZ gene. The resulting plasmids (pETS121, pETS122 and pETS123) were introduced into C. ammoniagenes by electroporation and the expression of β-galactosidase from lacZ fusions was measured by Western blotting.

Expressed β-galactosidase was detected from the nrdF and the ORF promoter regions (Fig. 2a; pETS121 and pETS122). Several clones of pETS123 were tested and surprisingly, no β-galactosidase was detected from the nrdHIE promoter region. We then decided to use the more sensitive technique of non-quantitative RT-PCR, using primers 13 and 14 to detect the lacZ mRNA transcript of the previous transcriptional fusions. As shown in Fig. 2(b), lacZ transcripts were detected in all three promoter fusions, indicating that all genes are transcribed from their own promoter.

**Growth-phase-dependent expression of nrdHIE, nrdF and the ORF**

The β-galactosidase activity of the nrdHIE, nrdF and ORF promoters was also studied throughout the C. ammoniagenes culture growth curve. The nrdF gene was expressed during the entire exponential growth phase and its transcription ceased when the cells reached the stationary phase. The highest level was observed at the beginning of the exponential phase, coinciding with the rapid replication of the bacterial chromosome (Fig. 3a). However, expression of the ORF followed an inverse pattern compared with the nrdF gene. During the exponential phase, the ORF β-galactosidase activity was relatively low, reaching the highest level at the end of this phase and remaining high throughout the stationary phase (Fig. 3a). As it was impossible to detect β-galactosidase activity in pETS123, we carried out RT-PCR to evaluate the nrdHIE transcripts throughout the culture growth curve. In Fig. 3(b), it can be seen how this promoter follows the same transcription pattern as observed for the nrdF gene, with the highest expression being achieved at the beginning of the exponential phase, followed by a decrease in the stationary phase.

**Different basal levels of nrdHIE and nrdF transcripts**

We used quantitative RT-PCR to establish the ratio of nrdHIE-specific to nrdF-specific messengers. Known amounts of a competitor transcript were mixed with total RNA preparations in such a way that the resulting amplified products were proportional to the relative amounts of native and competitor transcripts in the reaction (see Methods). Hybrid molecules are constituted by complementary strands of the competitor and wild-type molecules. Higher levels of nrdF mRNA were observed compared
Calculation of the relative amounts of mRNA levels revealed that nrdF transcription (0.472 pg ml⁻¹) was 9.1 times higher than that of nrdHIE (0.052 pg ml⁻¹).

Wild-type C. ammoniagenes was grown with the iron chelator DIP, with iron, with a high concentration of manganese, with hydrogen peroxide (H₂O₂) and in the presence of hydroxyurea for 40 min. Time-course analysis showed high levels of expression for this length of induction period. Total RNA was isolated and used as a template in RT-PCR experiments. cDNA from the 16S RNA gene was used to normalize the samples in these experiments, as transcript levels were not altered under any of the experimental growth conditions tested (Fig. 5, top). As shown in Fig. 5, transcription of nrdHIE and nrdF was greatly increased after exposure to hydroxyurea, manganese and H₂O₂. Significantly, mRNA levels for nrdHIE and nrdF increased 7.6- and 6.6-fold, respectively, in the culture treated with hydroxyurea, 5- and 6-fold with manganese, and 3.6- and 5.5-fold with H₂O₂. No variations in mRNA levels were observed when the cultures were treated with DIP or iron.

**nrde and nrdF are essential for aerobic growth of C. ammoniagenes**

To determine whether the nrdHIEF genes were the only RNR genes present in this micro-organism, we performed Southern-blot analysis on C. ammoniagenes wild-type chromosomal DNA using several probes from RNR classes from different bacterial species (see Methods). Genomic DNA was digested with BamHI, EcoRI, EcoRV, HindIII, MluI, SacI and SmaI and Southern-blot hybridizations were performed under both high- and low-stringency conditions. All Southern blots at any stringency revealed only a single band for the nrde, nrdF and nrdI genes, but...
no hybridization signals could be observed with nrdD, nrdI, nrdA or nrdB probes.

DNA constructs containing a kanamycin-resistance gene from the pK18mob plasmid together with the target genes were generated for insertional inactivation of nrdHIE and nrdF and used to transform C. ammoniagenes wild-type (see Methods). In three different experiments performed with the plasmids containing the nrdI and nrdF internal fragments, no kanamycin-resistant cells of C. ammoniagenes could be obtained on standard growth media, which strongly suggests that the nrdE and nrdF gene products are essential for growth. As a positive control, we were able to obtain mutants in the sugar transporter protein (STP) gene that was present upstream of the nrdH gene by using the same mutagenic strategy and plasmid pETS126 (data not shown).

**Organization of nrdEF genes in C. ammoniagenes and other species**

BLAST searches were performed with the finished and unfinished genome databases at the NCBI Microbial Genome BLAST website and GenBank (last date accessed 15 August 2002) by using C. ammoniagenes and S. typhimurium class Ib amino acid sequences. The Enterobacteriaceae and some Gram-positive bacterial species like Enterococcus faecalis and Lactococcus lactis contain the genes for the nrdEF operon arranged in the same manner (nrdH–nrdI–nrdE–nrdF) (Fig. 6). Nevertheless, there are several exceptions. Deinococcus radiodurans displays the usual arrangement of the operon, but the nrdH gene is absent (nrdI, nrdE and nrdF). Bacillus subtilis contains two copies of the nrdF operon; one of these also lacks the nrdH gene, and the other copy, of phage origin, has the whole structure, though organized in a slightly different way, with the end of the operon. Other Bacillus species like B. cereus and B. anthracis show the nrdI–nrdE–nrdF structure and nrdH is located elsewhere on the genome.

There is one group of bacteria that share an atypical nrdF because the translated protein lacks some of the amino acids needed for iron binding. The Mycoplasma species, with a peculiar structure of their unique class Ib nrd genes, nrdF–nrdI–nrdE, are included in this group. Streptococcus pyogenes and Streptococcus agalactiae contain this operon structure too, but with an extra class Ib operon without the nrdF gene and arranged as nrdH–nrdI–nrdE–nrdF. Staphylococcus epidermidis also has two copies of the nrdEF genes, one of these with the nrdI–nrdE–nrdF structure and the other one, as in B. subtilis of phage origin, with the nrdI–nrdE–nrdF structure. Staphylococcus aureus shows the nrdI–nrdE–nrdF structure (Masalha et al., 2001). Streptococcus mutans and Streptococcus pneumoniae show the nrdH–nrdE–nrdF structure plus an nrdI gene located elsewhere. C. diphtheriae contains two additional copies just like Strep. pyogenes, but the nrdE gene is absent in the nrdF–nrdI–nrdE copy. So far, all species examined have been found to contain at least the nrdIEF genomic organization but in the latter group of bacterial species, belonging to the Mycobacteria family, an intergenic region is present between nrdE and nrdF. In C. diphtheriae, C. ammoniagenes, Mycobacterium tuberculosis and Corynebacterium glutamicum nrdE is separated from nrdF by 862, 1171, 2707 and 3100 bp, respectively. In M. tuberculosis, an additional nrdF gene appears to be located elsewhere on the genome.

**DISCUSSION**

We have described the transcriptional and genetic regulation and the organization of the nrd genes encoding the active RNR class of C. ammoniagenes.

From our Southern blotting results, class Ib is the only class of RNR present in C. ammoniagenes and since nrdE, nrdF and nrdI probes hybridized to a single fragment in all digest, it is likely that this bacterium has a single copy of the genes that encode this class. Moreover, we were unable to isolate any mutant for either nrdE or nrdF, suggesting that these genes are essential and support bacterial growth, corroborating our previous biochemical data in which only class Ib activity was found in the protein extract (Fieschi et al., 1998).
We previously found five ORFs within this nrd region, four of them corresponding to well-known nrdHIEF genes, and one additional ORF between nrdF and nrdE (Fieschi et al., 1998). This particular arrangement of the nrdEF region deviates from the general structure of almost all class Ib operons (Fig. 6), but it is not atypical in the sense that it is shared with certain species from the mycobacterial family, including Corynebacterium and Mycobacterium species. In C. glutamicum only, there is one protein within this region homologous to the deduced protein encoded by the ORF found in the intergenic region of C. ammoniagenes. However, no similarities were found when comparing the intergenic regions in the other species of the Mycobacterium family. The function of this ORF remains unknown, although a BLAST search of sequence databases shows the highest similarities to known bacterial transcriptional factors, especially those belonging to the GntR family (Rigali et al., 2002). Our preliminary data indicate that this regulatory protein is not involved in the transcriptional regulation of nrdHIE or nrdF (unpublished results).

Transcriptional analysis of this region revealed three independent transcripts. nrdH, nrdI and nrdE are transcribed as a single mRNA, while two independent mRNAs were detected for the ORF and nrdF. No transcriptional linkage was found between nrdE and nrdF. By using lacZ fusions and mRNA lacZ detection we also demonstrated that the three mRNAs are transcribed from their own promoters. Immunodetection of the nrdHIE fusion was unsuccessful, probably due to the low expression level reached by this promoter, as seen in the quantification of the nrdF and nrdE basal levels. This system differs from those seen in previously studied class Ib RNRs (Jordan et al., 1996a), where all genes (nrdHIEF) were transcribed in a single mRNA. These findings raised a considerable number of questions about the regulation of the genes found within this region.

Previous studies showed that expression of RNR genes is cell-cycle dependent (Guittet et al., 2001; Jacobson & Fuchs, 1998), being maximal at the beginning of the exponential phase, where increasingly large amounts of dNTPs are required for DNA synthesis and chromosome replication, and decreasing through the late exponential and stationary phases. We have shown that the mRNA levels of C. ammoniagenes nrdE and nrdF are also down-regulated from exponential growth to the stationary phase, following the expected transcriptional pattern for RNR genes. Thus, a shared regulation mechanism is inferred for these two genes, despite their being transcribed from independent promoters. The ORF, however, is poorly transcribed during the exponential phase and reaches a peak at the end of this phase, remaining constant during stationary growth.

It is worth mentioning that even though the transcription of nrdHIE and nrdF follows the same pattern, transcription from the nrdF promoter is approximately 9 times higher than that from the nrdHIE promoter, which is very low as shown by competitive RT-PCR and inferred from our unsuccessful efforts to immunodetect the protein. Taking into account the need for equimolar amounts of both subunits to form an active holoenzyme, nrdHIE transcripts turn out to be the limiting factor for ribonucleotide reduction in C. ammoniagenes. Nevertheless, when the expression of a gene is studied at the mRNA level, it must be borne in mind that post-transcriptional or other gene-regulatory mechanisms may be present; this has been shown to be of great importance in mammalian and malaria parasite RNRs (Björklund et al., 1990; Breidbach et al., 2000).

As shown in Fig. 5, hydroxyurea, manganese and hydrogen peroxide appear to increase the transcription levels of nrdHIE and nrdF. Manganese induces nrdHIE and nrdF 5- and 6-fold, H₂O₂ 3-6- and 5-5-fold and hydroxyurea 7-6- and 6-6-fold, respectively. No induction was found in the presence either of iron or of the iron-chelating agent DIP.

Hydrogen peroxide induction of RNR has also been described in E. coli (Monje-Casas et al., 2001) and it seems a natural response of the cell to counteract DNA damage caused by reactive oxygen species. In E. coli, this induction has been thought to be involved in the oxidative stress response, but to date it has proved impossible to isolate any transcriptional regulator responsible for this effect. In E. coli, neither OxyRS nor SoxRS is involved in the regulation of the nrdEF genes. We can not exclude that hydrogen peroxide inhibits the radical of RNR and starves the cell for deoxyribonucleotides, thereby upregulating nrd gene expression.

Induction by hydroxyurea is a typical feature of class Ia RNRs (Fipula & Fuchs, 1978) and it is closely related to cell cycle inhibition. This compound scavenges the tyrosyl radical from the small subunit of class I RNR, inhibiting the overall activity of the enzyme. No dNTPs are produced and DNA synthesis is therefore blocked. Once again, the mechanisms involved in upregulating the transcription of these genes in response to hydroxyurea remain unknown.

The results presented in this study do not yet provide a satisfactory explanation for the transcriptional regulation of C. ammoniagenes class Ib RNR, and further studies are required to identify the proteins involved in induction by hydrogen peroxide and hydroxyurea. However, it is worth noting that, in these cases, DNA synthesis is altered either directly or indirectly. We postulate that a general regulator sensing an insufficient rate of DNA synthesis might either directly induce RNR expression or regulate the expression of intermediate regulators. This hypothesis has previously been postulated for E. coli class Ia RNR, where an SOS-independent induction of this enzyme was observed in response to DNA synthesis inhibition (Jordan et al., 1996a).

It is well known that C. ammoniagenes cell growth is strongly dependent on the presence of manganese in the culture.
medium (Oka et al., 1968) and it has also been suggested that many other bacteria, such as Treponema pallidum (Posey et al., 1999), utilize manganese and not iron for growth. In fact, manganese turns out to be a very important cation within the bacterial world, playing essential roles such as detoxifying a variety of reactive oxygen species and consequently, protecting bacteria from oxidative stress (Jakubovics & Jenkinson, 2001). In our study, we also showed that the effect of manganese on DNA replication should be looked for at the transcriptional regulation level instead of at the protein level of the class Ib RNR (Schimpff-Weiland et al., 1981; Willing et al., 1988).

If we take a careful look at the regions immediately upstream of both nrdH and nrdF, we are able, by comparing different C. glutamicum promoters (Fieschi et al., 1998), to identify a putative TATA box 111 bp upstream of nrdH (TATAGT) and 61 bp upstream of nrdF (TTATT). The putative −35 promoter sequence is located 132 bp upstream of nrdH (GGTTGCAG) and 87 bp upstream of nrdF (GGCAC).

According to the assumption that nrd promoters are directly or indirectly regulated by manganese and hydrogen peroxide, we examined the nucleotide sequences of these promoter regions by comparing them to OxyR-like and Fur-like binding motives. We identified a stretch of 24 nt located 112 bp upstream of nrdH (GCAGGTCAGC-GGCACAAATAG) and 52 bp upstream of nrdF (GAAGGGTTGGTTATTGGGTATGCT), which resembled the DtxR regulatory metal DNA sequences (Lee et al., 1997), suggesting a possible promoter region involved in the metal regulation of the nrd genes in C. ammoniagenes.

Fur and DtxR respond to the intracellular iron concentration and repress or activate the transcription of several genes (Hanke, 2001). Manganese homeostasis is controlled in a similar way by the DtxR-like protein Mntr (Jakubovics & Jenkinson, 2001). There is a high degree of similarity between the consensus sequences for these metal regulatory boxes and the stretch of 24 nt described in the nrd sequences, but very little is known about C. ammoniagenes iron- or manganese-regulated genes. This makes it particularly difficult to predict which kind of metal regulator accounts for our findings, but it seems clear that either one of them might be involved in regulating nrd transcription.

Iron and manganese have been proved to be very important ions in the bacterial world as they are involved in pathogenic processes. In this work we show, for the first time, that metal ions like manganese play a role in nrd regulation. The exact mechanisms of this regulation, yet to be deciphered, might provide important clues about the events that take place in a class Ib RNR-bearing pathogen during the course of an infection.

In this study we have shown that although both subunits are transcribed from independent promoters, there is a conserved mechanism of regulation that ensures their transcription under the same environmental conditions.

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