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A Rubrerythrin Operon and Nigerythrin Gene in *Desulfovibrio vulgaris* (Hildenborough)

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Rubrerythrin is a nonheme iron protein of unknown function isolated from *Desulfovibrio vulgaris* (Hildenborough). We have sequenced a 3.3-kbp *Sal*I fragment of *D. vulgaris* chromosomal DNA containing the rubrerythrin gene, *rbr*, identified additional open reading frames (ORFs) adjacent to *rbr*, and shown that these ORFs are part of a transcriptional unit containing *rbr*. One ORF, designated *fur*, lies just upstream of *rbr* and encodes a 128-amino-acid-residue protein which shows homology to Fur (ferric uptake regulatory) proteins from other purple bacteria. The other ORF, designated *rdl*, lies just downstream of *rbr* and encodes a 74-residue protein with significant sequence homology to rubredoxins but with a different number and spacing of cysteine residues. Overexpression of *rdl* in *Escherichia coli* yielded a protein, Rdl, which has spectroscopic properties and iron content consistent with one $Fe^{3+}(SCys)_4$ site per polypeptide but is clearly distinct from both rubrerythrin and a related protein, nigerythrin. Northern analysis indicated that *fur*, *rbr*, and *rdl* were each present on a transcript of 1.3 kb; i.e., these three genes are cotranscribed. Because *D. vulgaris* nigerythrin appears to be closely related to rubrerythrin, and its function is also unknown, we cloned and sequenced the gene encoding nigerythrin, *ngr*. The amino acid sequence of nigerythrin is 33% identical to that of rubrerythrin, and all residues which furnish iron ligands to both the FeS_4 and diiron-oxo sites in rubrerythrin are conserved in nigerythrin. Despite the close resemblance of these two proteins, *ngr* was found to be no closer than 7 kb to *rbr* on the *D. vulgaris* chromosome, and Northern analysis showed that, in contrast to *rbr*, *ngr* is not cotranscribed with other genes. Possible redox-linked functions for rubrerythrin and nigerythrin in iron homeostasis are proposed.

Rubrerythrin (Rr) is one of a large number of nonheme iron proteins found in anaerobic sulfate-reducing bacteria (16, 28). Rr was isolated from *Desulfovibrio vulgaris* (Hildenborough) as a 44-kDa homodimer which exhibited spectroscopic signatures characteristic of two distinct types of iron sites: one rubredoxin-like FeS_4 site and one nonsulfur, oxo-bridged diiron site. The crystal structure of recombinant *D. vulgaris* Rr (10) revealed that each subunit is folded into two domains: an N-terminal four-helix bundle surrounding the oxo-bridged diiron site, and a smaller C-terminal rubredoxin-like protein fold surrounding the FeS_4 site. Rr can be structurally classified as a member of a continually expanding class of so-called diiron-oxo proteins (20, 30). This class of proteins contains an oxo- or hydroxo-bridged diiron site connected to the protein by carboxylate and histidine ligands and embedded within a four-helix bundle protein fold. Other members of this class utilize O_2 for functions ranging from tyrosyl radical generation and hydrocarbon hydroxylation in prokaryotes, fatty acyl desaturation in plants, and reversible O_2 binding in invertebrates (20). The iron storage proteins ferritin and bacterioferritin apparently have metastable diiron sites similar to those found in diiron-oxo proteins (20), and the peptide backbone structure of the four-helix bundle in Rr is nearly superimposable on those of the ferritin and bacterioferritin subunits (10, 30). Subsequent to the isolation of Rr, a slightly larger homodimeric protein named nigerythrin (Ngr) (due to its black color), which contained spectroscopic signatures for the same two types of iron sites, was isolated from the same *Desulfovibrio* species (33). No other known proteins have the combination of FeS_4 and diiron-oxo sites found in Rr and Ngr.

The physiological roles of Rr and Ngr have not been determined. The unusually positive reduction potentials of both the diiron-oxo and FeS_4 sites (>200 mV versus the normal hydrogen electrode [14, 33]) make it difficult to place Rr or Ngr within any known electron transport chain of anaerobic sulfate-reducing bacteria. Based on matching of reduction potentials, a trithionate reductase function for Rr has been suggested (16) but never demonstrated. Since FeS_4 sites in rubredoxins are not known to function as catalysts other than of electron transfer (44), the diiron site presumably holds the key to the function of Rr. The low overall amino acid sequence homology between Rr and other known diiron-oxo proteins (10, 21) suggests no obvious evolutionary relationship or biological activity. Rr and Ngr lack activities characteristic of other diiron-oxo proteins and also have no catalase or phosphatase activities (14, 33). (The pyrophosphatase activity reported for Rr [24] has not been reproduced [21, 33].) Recombinant Rr does have ferroxidase activity (i.e., the ability to catalyze oxidation of Fe^{2+} to Fe^{3+} by O_2) in vitro, with specific activity comparable to those of apoferritins (5). However, ferritins and bacterioferritins form 24-mer protein shells that solubilize and store the polyiron(III) oxyhydroxide aggregates resulting from iron oxidation, whereas Rr appears to form only a homodimer in solution (or homotetramer in the crystal). Strict anaerobes have only rarely been found to have ferritins (40), presumably because only a small proportion of Fe^{3+} is present in reducing environments.

Rr from another anaerobic bacterium, *Clostridium perfringens*, has recently been reported (23). This Rr was purified as

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TABLE 1. Bacterial strains and plasmids

Bacterium or plasmid(s)	Genotype or relevant characteristic(s)	Source and/or reference
D. vulgaris (Hildenborough)		Laboratory stock, University of Georgia Fermentation Facililty; 55
E. coli		
JM109	e14 (*mcrA*) *recA1 endA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^+) *supE44 relA1* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q}*ZΔM15*]	Laboratory stock
DH5α	Δ(*lacZYA-argF*)*U169* ϕ80d *lacZΔM15*) *deoR recA1 endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 gyrA96 relA1*	Life Technologies, Inc. (Gaithersburg, Md.)
K-12 71/18	*supE thi*Δ(*lac-proAB*) [F′ *proAB*$^+$ *lacI*^{q} *lacZΔM15*]	27
BL21(DE3)	F$^-$ *dcm ompT hsdR17* (r_K^- m_K^+) *supE44 relA1*	Novagen, Inc. (Madison, Wis.); 49
Plasmids		
pUC18	Cloning vector, Ap^r	Laboratory stock
pT7-7	Expression vector, Ap^r	50
pNS560	pUC18 derivative carrying the 4.5-kbp *Eco*RI/*Pst*I fragment from *D. vulgaris* chromosomal DNA containing *ngr*[a]	This work
pNS-S-P	pUC18 derivative carrying the 1.8-kbp *Sal*I/*Pst*I fragment from *D. vulgaris* chromosomal DNA containing *ngr*[a]	This work
pNS-S-S	pUC18 derivative carrying the 1-kbp *Sal*I/*Sal*I fragment from *D. vulgaris* chromosomal DNA upstream of *ngr*[a]	This work
pNS1	pT7-7 derivative carrying the *D. vulgaris ngr* gene inserted into the *Nde*I/*Bam*HI sites	This work
pBP96-4	pUC9 derivative carrying 7-kbp *Pst*I fragment from *D. vulgaris* (Hildenborough) chromosomal DNA containing *rbr*	34
pRG101 and pRG103	pUC18 derivatives containing 3.3-kbp *Sal*I fragment from pBP96-4 in both orientations	This work
pRG104	pRG101 derivative with the 1,076-bp *Sal*I/*Eco*RI fragment[b]	This work
pRG105	pRG101 derivative with the 813-bp *Sal*I/*Bgl*II fragment[b]	This work
pRG106	pRG101 derivative with the 219-bp *Sal*I/*Stu*I fragment[b]	This work
pRG107	pRG101 derivative with the 404-bp *Sal*I/*Stu*I fragment[b]	This work
pRG108	pRG103 derivative with the 1,303-bp *Sma*I/*Sal*I fragment[b]	This work
pRG109	pRG103 derivative with the 133-bp *Sma*I/*Sal*I fragment[b]	This work
pRrOR1	pT7-7 derivative containing *D. vulgaris rdl* inserted into the *Nde*I/*Bam*HI sites	This work
pC68A	pRrORF1 derivative containing the CA→GC mutation in *rdl* corresponding to the C68A mutation in Rdl	This work
pC68A,C53A	pc68A derivative containing the CA→GC mutation in *rdl* corresponding to the C53A mutation in Rdl	This work

[a] Refer to Fig. 6 for approximate locations of restriction sites.
[b] Refer to Fig. 1 for approximate locations of restriction sites.

a superoxide dismutase (SOD)-active fraction from cellular extracts of *C. perfringens* grown under oxidative stress conditions. The gene encoding *C. perfringens* Rr was subsequently cloned and was able to partially restore oxidative stress resistance when expressed in an *Escherichia coli sodAB* strain. However, these results could not establish whether SOD represents the native physiological function of *C. perfringens* Rr.

Prickril et al. (34) determined that a 7-kbp *Pst*I restriction fragment of *D. vulgaris* (Hildenborough) chromosomal DNA contained the Rr-encoding gene, *rbr*, which they sequenced and further localized to a 3.3-kbp *Sal*I restriction fragment. As part of our effort to understand the biological function of Rr, we have sequenced the entire 3.3-kb *Sal*I fragment, identified additional open reading frames (ORFs) adjacent to *rbr*, and shown that these ORFs are part of a transcriptional unit containing *rbr*. We have carried out similar studies on the gene encoding *D. vulgaris* Ngr. We present these results along with some characterization of the gene products and discuss their possible functional implications.

MATERIALS AND METHODS

Strains, plasmids, media, and growth conditions. Strains and plasmids used are described in Table 1. Luria-Bertani (LB) medium supplemented when necessary with 100 μg of ampicillin/ml (LB-ampicillin) was used for growth of *E. coli* strains. *D. vulgaris* (Hildenborough) was grown in lactate-sulfate medium in either 10-ml tubes or 3-liter flasks for approximately 18 h at 37°C, using standard anaerobic Hungate techniques (55). The lactate-sulfate medium contained 6.7 mM sodium lactate, 40 mM ammonium chloride, 10 mM magnesium sulfate, 3 mM potassium phosphate, 30 mM sodium sulfate, 2 mM calcium chloride, 36 μM ferrous sulfate heptahydrate, 0.6 mM cysteine hydrochloride, 1 mM sodium sulfide, 1% (wt/vol) yeast extract (Difco), and 1% (vol/vol) Wolfe's trace mineral solution (38), modified by addition of 0.1 g of $NiCl_2 \cdot 6H_2O$/liter (pH 7.3).

General molecular biology methods. Molecular biology procedures were carried out as described in reference 41 or 2. Restriction enzymes were from either Boehringer Mannheim (Indianapolis, Ind.) or New England Biolabs (Beverly, Mass.) and were used with vendor-supplied buffers. All solutions were prepared in MilliQ (Millipore Corp., Bedford, Mass.)-treated water. Oligonucleotides were purchased from Integrated DNA Technologies Inc. (Doralville, Iowa). For RNA isolation from *D. vulgaris* (Hildenborough), cells from a 10-ml culture were resuspended in 100 μl of TE (10 mM Tris-hydrochloride, 1 mM EDTA [pH 8]) containing 400 μg of lysozyme/ml. Total RNA was isolated by using a Qiagen RNEasy RNA isolation kit (Qiagen Inc., Santa Clarita, Calif.), resuspended in 30 μl of diethylpyrocarbonate-treated water, and immediately subjected to Northern analysis (as described below). Digoxygenin (dig)-labeled RNA molecular weight markers were purchased from Boehringer Mannheim. Genomic DNA from *D. vulgaris* (Hildenborough) was isolated and purified from a 1-liter culture as described by Robson et al. (39). The genomic DNA was resuspended in TE. PCRs were carried out in 1× PCR buffer with 1.5 mM $MgCl_2$ (Boehringer Mannheim), 200 μM deoxynucleoside triphosphate, 0.5 μM primers (Table 2), and 2.5 U of *Taq* DNA polymerase (Boehringer Mannheim or Promega [Madison, Wis.]) in a total volume of 50 μl. The PCR amplification procedure consisted of first heating template DNA and primer oligonucleotides at 94°C for 5 min and then, after addition of the remaining reaction components, running 30 cycles of 94°C for 1 min, either 37°C (for *rdl*, *rbr*, or *fur*) or 60°C (for *ngr*) for 1

TABLE 2. Primers used for PCRs

Primer name	Nucleotide sequence[a]
RrDS-for	5′ TATAcatatgGCGAACCCTGAAGAC 3′
RrDS-rev	5′ TAGAggatccTCACTTGTCGGTCGGC ATCT 3′
ORFMUT1-B	5′ TAGAggatccTCACTTGTCGGTCGGC ATCTCGgcTTGCG 3′
Mut2.R	5′ GCGGAAGgcCTTCTTGGTGGCTTT 3′
Mut2.F	5′ AAGAAGgcCTTCCGCCCGTTGGCT 3′
Ngr-for	5′ TATAcatatgAAAGTCAGAGCACAG 3′
Ngr-rev	5′ TAGAggatccTCAATAGGCTGTAA 3′
Fur.for	5′ TATAcatatgTCTCAAACCCGTA 3′
Fur.rev	5′ TAGAggatccTCAGCTATGTGCTGT 3′

[a] *Nde*I (catatg) and *Bam*HI (ggatcc) restriction sites are shown in lowercase. Underlined regions duplicate gene sequences with mutated nucleotides shown in underlined lowercase, as indicated in the text.

min, and 72°C for 2 min. Plasmids were purified by using Qiagen Tip-100 columns. Nucleotide sequences of genes in the plasmids generated in this work were verified by DNA sequencing, which was carried out in the Molecular Genetics Instrumentation Facility at the University of Georgia.

Overexpression of recombinant proteins. Recombinant *D. vulgaris* (Hildenborough) proteins were overexpressed in *E. coli* BL21(DE3) from the genes inserted into the *Nde*I and *Bam*HI sites of pT7-7, as described by Gupta et al. (14).

Nucleotide sequencing of regions flanking *rbr*. Plasmids pRG101 and pRG103-109 (Table 1) were used for sequencing.

Cloning and mutations of *rdl*. The *rdl* (rubridoxin-like) gene was amplified in a PCR using *Pst*I-digested pBP96-4 as the template and oligonucleotides RrDS-for and RrDS-rev as primers. The underlined nucleotide sequences in RrDS-for and RrDS-rev (Table 2) duplicate the 5′ end and complementary 3′ end, respectively, of *rdl*. The 224-bp PCR product was purified by using a Wizard PCR Prep kit (Promega) and then ligated into the *Nde*I/*Bam*HI sites of pT7-7 by using T4 ligase. The ligation mixture was transformed into *E. coli* K-12 71/18, yielding pRrORF1.

A C68A mutation of *rdl* was obtained by using PCR with pRrORF1 as the template and RrDS-for and ORFMUT1-B as primers. The 30-nucleotide sequence following the *Bam*HI site in ORFMUT1-B (underlined in Table 2) duplicates the complementary 3′ end of *rdl* except that nucleotides 23 and 24 were changed from CA to GC. Ligation of the PCR product to pT7-7 generated plasmid pC68A. The C53A,C68A mutation of *rdl* was constructed by overlap extension PCR (36). Using pC68A as the template, we performed two separate PCRs with primer pairs RrDS-for–Mut2.R and Mut2.F–Rrds-rev. Both Mut2.R and Mut2.F duplicate internal sequences of *rdl* and the first 15 bases are complementary to each other, with nucleotides in lowercase being those changed to achieve the C53A mutation (Table 2). The two PCR products (ca. 175 and 80 bp) were spliced together in a second PCR using 1 μl of each PCR product solution as the template and RrDs-for and RrDs-rev as primers. Ligation of the resulting 224-bp PCR product into *Nde*I/*Bam*HI sites of pT7-7 resulted in plasmid pC53A,C68A.

Isolation and purification of recombinant Rdl. Plasmid pRrORF1 was transformed into *E. coli* BL21(DE3), and protein overexpression was induced as described above. The cells from 1 liter of induced *E. coli* BL21(DE3)[pRrORF1] were lysed by three freeze (−80°C)-thaw (room temperature) cycles (19). After the final thaw, the cells were resuspended in 50 ml of 50 mM Tris-HCl–1 mM EDTA (pH 8.0) and then centrifuged at 15,000 × *g* for 45 min. The supernatant was concentrated to approximately 10 ml under argon pressure in a 50-ml Amicon cell equipped with a YM3 membrane. The concentrated, red-colored supernatant was applied to a Superose-12 column (1.6 by 50 cm; Pharmacia LKB) equilibrated with 50 mM Tris-HCl–1 M NaCl (pH 8.0), and the protein was eluted with the same buffer. The red-colored fractions were collected and desalted by multiple dilutions and concentrations in an Amicon cell, using 50 mM Tris-HCl (pH 8.0). The red, iron-containing Rdl (iron Rdl) and a colorless fraction, presumed to be zinc-containing Rdl (zinc Rdl), were separated from each other by applying the desalted protein solution to a MonoQ anion-exchange column (HR10/10; Pharmacia LKB) equilibrated with 50 mM Tris-HCl (pH 8.0) and eluting with a 0 to 25% of 1 M NaCl gradient in the same buffer. The iron Rdl eluted at approximately 20%, and the putative zinc Rdl eluted at approximately 25% in the salt gradient. The purified yield of iron Rdl was approximately 20 mg/liter of culture. The same procedure was used to purify C53A,C68A Rdl from *E. coli* BL21(DE3)[pC53A,C68A]. The purified recombinant wild-type or C53A,C68A Rdl gave a single band upon electrophoresis in Tricine sodium dodecyl sulfate-polyacrylamide gels (Tricine SDS-PAGE) (42). The purified proteins were stored at −80°C.

Cloning and nucleotide sequencing of *ngr* and adjacent regions. Based on the published N-terminal amino acid sequence, MKVRAQVPTVKNATN, for Ngr (33), a degenerate oligonucleotide probe, designated Ngr-DF, with the sequence 5′ GCNGADGTNCCNACNGTNAADAAYGC 3′ was synthesized and 3′ end labeled with dig-ddUTP according to the protocol for the Genius system (Boehringer Mannheim). A Southern blotting performed as specified in the Genius system user's guide demonstrated that Ngr-DF hybridized to a 4.5-kbp *Eco*RI/*Pst*I restriction fragment of *D. vulgaris* chromosomal DNA. Therefore, fragments of *Eco*RI/*Pst*I-cut *D. vulgaris* chromosomal DNA in the 4- to 5-kbp range were isolated by size fractionation on a 0.8% low-melting-temperature agarose gel, recovered by electroelution, and ligated into pUC18. The ligation mixture was transformed into *E. coli* DH5α, and individual white colonies were picked and cultured at 37°C in 1.5 ml of LB-ampicillin. Plasmids were purified by using the alkaline lysis procedure from six combined 1.5-ml cultures, and 5-μl aliquots of the pooled plasmid preparations were screened for the presence of the 4.5-kbp *Eco*RI/*Pst*I fragment containing *ngr* by Southern hybridization to dig-labeled Ngr-DF. Screening of 100 plasmid pools resulted in the isolation of three plasmids containing the 4.5-kbp *Eco*RI/*Pst*I fragment. Further subcloning of restriction fragments from one of these positive plasmids, pNS560, into pUC18, followed by restriction digestion and Southern hybridization to dig-labeled Ngr-DF generated the restriction map shown in Fig. 6 of a 2.8-kbp *Sal*I/*Pst*I fragment containing *ngr*. The *ngr* gene was further localized to a 1.8-kbp *Sal*I/*Pst*I fragment in plasmid pNS-S-P, which was sequenced. The 5′ end of *ngr* was located approximately 50 bp downstream of the *Sal*I site. The connecting upstream 1-kbp *Sal*I/*Sal*I fragment was subcloned into pUC18 to generate pNS-S-S and similarly sequenced. Finally, the connection between the 1-kbp *Sal*I/*Sal*I and 1.8-kbp *Sal*I/*Pst*I fragments was made by sequencing this region in pNS560.

Cloning of *ngr* into pT7-7. The nucleotide sequence encoding *ngr* was amplified from pNS560 by using PCR with primers Ngr-for and Ngr-rev. The PCR product was purified and ligated into pT7-7, resulting in plasmid pNS1, which was transformed into *E. coli* BL21(DE3).

Isolation and purification of Ngr. Induction of Ngr overexpression from *E. coli* BL21(DE3)[pNS1] was carried out as described above. All procedures were carried out at room temperature unless indicated otherwise. The induced cells from two combined 1-liter cultures were washed once with 250 ml of 50 mM Tris-HCl–2 mM EDTA (pH 8.0) and then resuspended in 30 ml of the same buffer. One milliliter of lysozyme (10 mg/ml) and 10 ml of 1% (vol/vol) Triton X-100 were then added. After incubation at 30°C for 15 min, the mixture was sonicated on ice for 1 min with 1-s bursts at 0.5-s intervals, using a Sonic Dismembrator (Fisher Scientific). The grey pellet from the resulting cell lysate was collected by centrifugation at 12,000 × *g* for 30 min at 4°C. Tricine SDS-PAGE analysis indicated that the majority of this pellet consisted of an overexpressed protein having the expected molecular weight of the Ngr subunit. The pellet was suspended in 6 ml of 3 M guanidine hydrochloride, and iron was incorporated into the protein as described by Gupta et al. for Rr (14) except that the buffer was 50 mM HEPES (pH 7.5). The final supernatant was applied to a small (30- to 40-ml) QAE-Sephadex G-25 (Pharmacia LKB) column equilibrated with 20 mM Tris-HCl (pH 8.0). Most of the Ngr (black-colored fractions) passed through this column, and the remainder was eluted with the same buffer. These Ngr fractions were combined and concentrated by ultrafiltration. Final purification was carried out by gel filtration on a Superose-12 fast protein liquid chromatography column (1.6 by 50 cm; Pharmacia LKB) equilibrated with 50 mM HEPES–200 mM sodium sulfate (pH 7.0). The yield of Ngr after this protocol was approximately 20 mg/liter of culture, based on the published extinction coefficient at 485 nm (33).

Northern analyses. The probes used for Northern analyses of *fur*, *rbr*, *rdl*, and *ngr* consisted of the corresponding dig-labeled genes, which were prepared by incorporating dig-labeled deoxynucleoside triphosphates (Boehringer Mannheim) in a PCR amplification. *Pst*I-digested pBP96-4 was used as the template, and the pairs of primers Fur.for-Fur.rev, DMK4-DMK5 (14), and RrDS-for–RrDS-rev were used to amplify the *fur*, *rbr*, and *rdl* genes, respectively. A dig-labeled *ngr* probe was similarly prepared, using pNS560 as the template and Ngr-for and Ngr-rev as primers. Northern analyses of *D. vulgaris* RNA with these probes were performed essentially according to the Genius system protocol (Boehringer Mannheim). The RNA was transferred to nylon membranes (Nytran; Schleicher & Schuell) by using a Schleicher & Schuell Turboblotter and cross-linked on the membrane by using a UV cross-linker (FB-UVXL-1000; Fisher Scientific). The membranes were then prehybridized in high-SDS buffer (0.75 M NaCl, 75 mM sodium citrate, 2.0% blocking reagent, 50 mM Na_2PO_4, 0.1% *N*-lauryl sarcosine, 7% SDS, 50% formamide) for 1 h at 46°C (*fur* probe), 1 h at 50°C (*rbr* or *rdl* probe), or 2 h at 55°C (*ngr* probe). The membranes were then hybridized in high-SDS buffer with 50 ng of dig-labeled probe/ml for approximately 15 h at the prehybridizing temperatures. Hybridization to the anti-dig-alkaline phosphatase and equilibration with LumiPhos 530 solution were done according to the Genius system protocol. After 15 min of equilibration at 37°C, the membranes were exposed to film for 15 to 45 min.

Chemical and spectroscopic characterization of Rdl and Ngr. UV/visible (vis) absorption spectra were recorded on a Shimadzu UV-210PC spectrophotometer, using 1-cm-path-length quartz cuvettes. X-band electron paramagnetic resonance (EPR) spectra of 200-μl protein samples which had been frozen in a liquid N_2 bath were recorded at various temperatures on a Bruker ESP-300E spectrophotometer fitted with an Oxford Instruments ESR-9 continuous-flow liquid helium cryostat. The molecular weight of iron Rdl was determined by mass spectrometry using a Perkin-Elmer Sciex API-1 plus quadrupole mass spectrom-

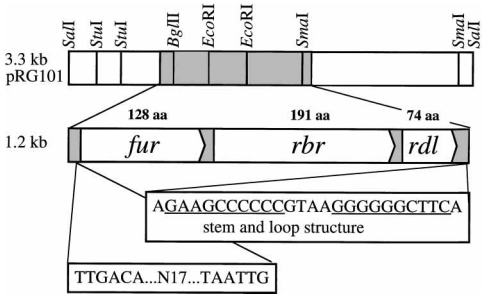

FIG. 1. Restriction and ORF map of the 3.3-kbp *Sal*I restriction fragment of *D. vulgaris* (Hildenborough) chromosomal DNA containing *rbr* in pRG101. The 1.2-kbp region (shaded) extending from the putative RNA polymerase promoter upstream of *fur* to the putative stem-loop structure downstream of *rdl* is expanded to show the relative sizes and locations of *fur*, *rbr*, and *rdl* and their gene products (aa, amino acid residues) in the three-gene cluster.

eter with an atmospheric pressure electrospray ionization source in the Mass Spectrometry Facility at the University of Georgia. The Rdl was diluted in 50:50 (vol/vol) acetonitrile-H_2O and introduced into the spectrometer via loop injection. A molar absorption coefficient at 485 nm was determined for the iron Rdl by averaging the quantities of alanine, proline, valine, threonine, and tyrosine from three quantitative amino acid analyses performed at the Harvard Microchemistry Facility at Harvard University, Cambridge, Mass., and using the amino acid composition inferred from the nucleotide sequence (Fig. 3). Metal contents of iron and zinc Rdl were determined by inductively coupled plasma-atomic emission spectrometry in the Chemical Analysis Laboratory at the University of Georgia. An N-terminal amino acid sequence of iron Rdl was determined at the Harvard Microchemistry Facility. Solution molecular weights of iron Rdl and Ngr were determined by gel filtration using the Superose-12 gel filtration column that had been used to purify these proteins. The column was calibrated with bovine serum albumin (molecular weight, 66,000), carbonic anhydrase (29,000), horse heart cytochrome *c* (12,400), and aprotinin (6,500). Ferroxidase activity of recombinant Ngr was measured as described by Bonomi et al. (5) for Rr from the rate of absorbance increase at 315 nm following addition of 0.18 to 4 nmol of Ngr to 1 ml of air-saturated buffer (50 mM HEPES, 200 mM sodium sulfate [pH 7.0]) containing 120 μM ferrous ammonium sulfate.

Sequence analyses. Homologous sequences in the GenBank database were found by using the BLAST algorithm (1). Sequences were aligned by using either the PILEUP or GAP algorithm in the Genetics Computer Group software package (version 8, 1994).

Nucleotide sequence accession numbers. The nucleotide sequence reported for *rbr*, *fur*, and *rdl* appears in the GenBank sequence database (3) under accession no. U82323. The nucleotide sequence of the 2.8-kbp *Sal*I/*Pst*I fragment containing *ngr* appears in the GenBank database under accession no. U71215.

RESULTS

Organization of ORFs flanking *rbr*. In the 3.3-kbp *Sal*I fragment of *D. vulgaris* (Hildenborough) chromosomal DNA which contains *rbr*, we identified and sequenced two additional complete ORFs, *fur* and *rdl*. These additional ORFs are transcribed in the same direction as *rbr* (Fig. 1). Sequences resembling the *E. coli* σ^{70} RNA polymerase −35 and −10 recognition regions lie 54 to 35 bp upstream of the *fur* start codon. A predicted stem-loop structure, perhaps signaling transcription termination, occurs 7 bp following the *rdl* stop codon (Fig. 1).

A *fur*-like gene upstream of *rbr*. An ORF occurring 431 to 44 bp upstream of the *rbr* start codon encodes a putative protein of 128 amino acid residues and is preceded by a ribosome binding site (GGAGG) 10 to 6 bp upstream of the start codon. Its designation as *fur* rests on the 24 to 30% sequence identity (47 to 55% sequence similarity) of the encoded protein to Fur (ferric uptake regulatory) proteins found in several other purple bacteria (Fig. 2). The putative *D. vulgaris* Fur-like protein is significantly shorter than the others (which range from 134 to 157 residues), with most of the missing residues occurring at the N and C termini, when aligned as shown in Fig. 2. Molecular biological evidence indicates that the N-terminal region (approximately residues 1 to 90) of *E. coli* Fur is involved in DNA binding (29, 47). Fur requires a divalent metal ion as corepressor, but no metal ligand residues have been conclusively identified. However, an HHXHXXCXXCG motif in the C-terminal region, which has been proposed as a potential metal binding sequence in other Fur proteins (31), is conserved in the putative *D. vulgaris* Fur. We have so far obtained only inclusion bodies from attempts to overexpress the *D. vulgaris* *fur* in *E. coli* (43).

Identification of *rdl* downstream of *rbr*. The start codon for the gene identified as *rdl* occurs 19 bp downstream of the *rbr* stop codon, is preceded by a ribosome binding site (GGGAGG) 10 to 5 bp upstream of the start codon, and encodes a protein of 74 amino acid residues. The amino acid sequence of the putative gene product, Rdl, is homologous to that of *D. vulgaris* rubredoxin (Fig. 3). In rubredoxins, iron ligands are furnished by four cysteine residues in a consensus $CX_2CX_{29}CX_2C$ motif (44). The predicted amino acid sequence of Rdl shows the first four of the six cysteine residues (21, 22, 34, 35) in the same motif except that the first two cysteine residues in Rdl are separated by four rather than two residues. Rubredoxins are also typically about 20 residues shorter than the inferred Rdl sequence, with most of the additional residues in Rdl occurring at the C terminus (44). A BLAST search of GenBank revealed no other sequences among the top 100 scores which duplicate the spacing of any four sequentially adjacent cysteine residues in Rdl.

The *D. vulgaris* Rdl was overexpressed in *E. coli* as a mixture of red, iron Rdl, and a colorless fraction, presumed to be zinc Rdl. This presumption is based on metal analyses after separation of the two fractions and the fact that iron Rdl and the zinc-containing protein migrated identically on SDS-polyacrylamide gels. Using the ε_{485} listed below, metal analyses of two iron Rdl samples yielded 1.09 and 1.12 mol of Fe/mol of protein and negligible quantities of other transition metals, whereas the putative zinc Rdl contained only zinc in detectable quantities. The fact that these iron and zinc forms could be separated by anion-exchange chromatography is apparently due to the 3+ and 2+ charges on the metal ions in the respective proteins, and these results parallel those obtained upon overexpression of rubredoxins in *E. coli* (37). We are unaware of any reports of zinc rubredoxin isolated from the parent organism, and we presume (as is presumed for rubredoxins) that the iron Rdl is the functional form in vivo. Recombinant iron Rdl was analyzed by mass spectrometry, and the resulting molecular weight, 8,415, agrees very well with that calculated from the inferred amino acid composition, 8,418, not including the N-terminal methionine residue or the metal ion (the latter presumably being lost either in the 50% acetonitrile solvent used for mass spectrometry or during electrospray ionization). N-terminal sequencing of the iron Rdl yielded an amino acid sequence, ANPED, for the first five residues, which is identical to that encoded by *rdl*, a finding which confirms the absence of an N-terminal methionine. Gel filtration of Rdl yielded a molecular weight of 12,000. Other small ferredoxins and rubredoxins are known to run at anomalously high molecular weights on gel filtration columns even with high levels of salt for unexplained reasons (4, 45). The UV/vis absorption spectrum of iron Rdl (Fig. 4) shows peaks at 350, 485, and 590 nm, which deviate only slightly from those characteristic of $Fe^{3+}(SCys)_4$ sites in rubredoxins (25, 37). An extinction coefficient at 485 nm of 8,400 M^{-1} cm^{-1} was determined for iron Rdl, which is close to the ε_{490} of 8,850 M^{-1} cm^{-1} for *Clostridium pasteurianum* rubredoxin (25). These very minor differences in UV/vis absorption characteristics between iron Rdl and rubredoxin are consistent with small perturbations of the $Fe^{3+}(SCys)_4$ sites due to a change in sequence spacing of the

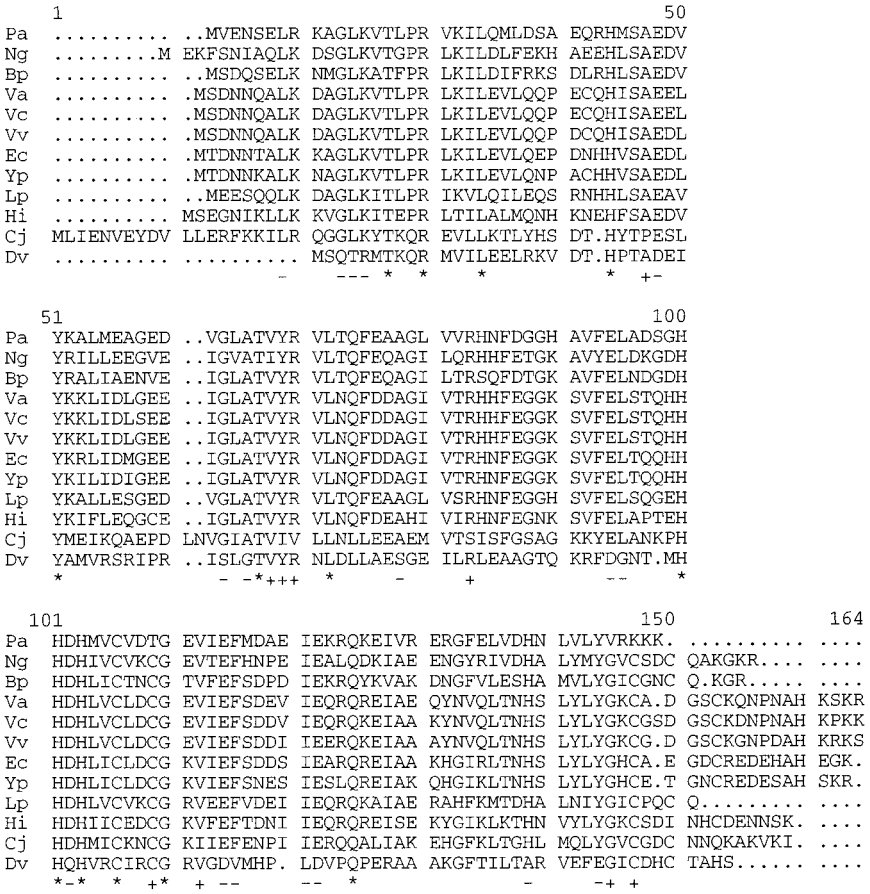

FIG. 2. Amino acid sequence alignment using PILEUP of Fur proteins from other purple bacteria with the *D. vulgaris* Fur-like ORF (Dv) determined in this work. Fur sequences are from the following organisms: *Pseudomonas aeruginosa* (Pa; GenBank accession no. L00604), *Neisseria gonorrhoeae* (Ng; L11361), *Bordella pertussis* (Bp; L31851), *Vibrio anguillarum* (Va; L19717), *Vibrio cholerae* (Vc; m85154), *Vibrio vulnificus* (Vv; L06428), *E. coli* (Ec; P06975), *Yersinia pestis* (Yp; Z12101), *Legionella pneumophila* (Lp; U06072), *Haemophilus influenzae* (Hi; U32704), and *Campylobacter jejuni* (Cp; X78965). *, conserved residue; +, residue conserved in all but one sequence; −, residue conserved in all but the *D. vulgaris* sequence.

cysteine ligand residues. Other potential iron ligand residues either are not present in Rdl (His) or would result in an iron site having visible absorption spectra (Ser, Asp, Glu) very different from that observed for iron Rdl or wild-type rubredoxin (25, 46).

Given the alignment of cysteine residues shown in Fig. 3 and the evidence presented above for a single $Fe^{3+}(SCys)_4$ site in each iron Rdl polypeptide, we supposed that Cys 9, 14, 44, and 47 furnish iron ligands, whereas the remaining two Cys residues, 58 and 63, do not. Consistent with this hypothesis, the UV/vis spectrum of C53A,C68A iron Rdl, in which Cys 58 and 63 are mutated to Ala, is not significantly different from that of the wild-type iron Rdl (Fig. 3).

Detection of a *fur-rbr-rdl* transcript. The proximity of the *fur*, *rbr*, and *rdl* genes to each other together with the putative RNA polymerase promoter upstream of *fur* and transcription termination signal downstream of *rdl* suggested to us that these three genes could be cotranscribed. This hypothesis was tested by Northern analysis of *D. vulgaris* total RNA. Probing separately with dig-labeled *fur*, *rbr*, and *rdl* revealed a 1.3-kb transcript in each case (Fig. 5). This size is within experimental error of the 1,314 bp expected for a *fur-rbr-rdl* transcript beginning at the putative RNA polymerase promoter sequence upstream of *fur* and ending at the putative stem-loop sequence downstream of *rdl*. These results indicate that the *fur*, *rbr*, and *rdl* genes are cotranscribed. A second RNA band of 0.8 kb was identified in Northern blots probed with *rbr* and *rdl*. The intensity of this smaller band was very much lower in Northern blots probed with *fur* (Fig. 5). This size and pattern of hybridization intensities are consistent with a second transcript containing only *rbr* and *rdl* (there being 828 bp from the *rbr* start to the beginning of the putative stem-loop structure downstream of *rdl*). If this is so, the shorter transcript appears to predominate over the larger one, based on their relative intensities in the Northern blots. We have found no homology to the *E. coli* σ^{70}-type RNA polymerase promoter sequence either in the intergenic region upstream of *rbr* or within the C-terminal

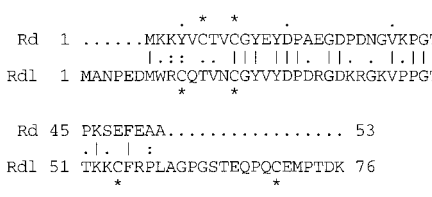

FIG. 3. Alignment of amino acid sequences of *D. vulgaris* Rdl (this work) and *D. vulgaris* rubredoxin (6), using the GAP algorithm. Cysteine residues are starred above or below the sequences. Symbols between the sequences indicate conservation (|) or decreasing similarity (: and .) of the aligned residues.

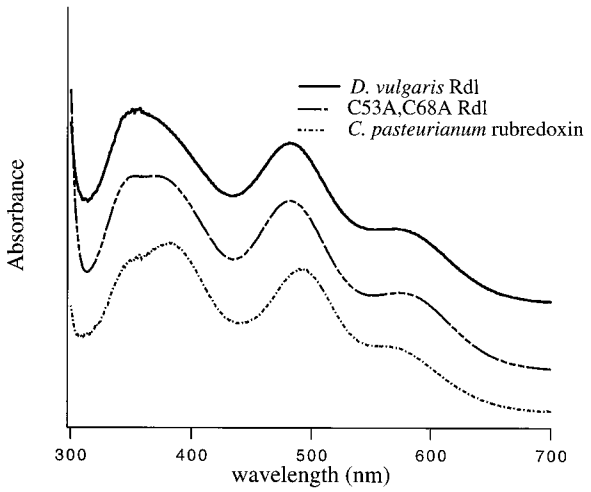

FIG. 4. UV/vis absorption spectra of *D. vulgaris* wild-type and C53A,C68A iron Rdl and *C. pasteurianum* iron rubredoxin (37).

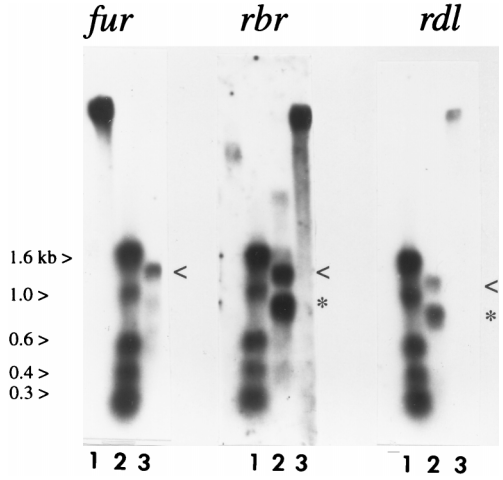

FIG. 5. Northern analysis of *D. vulgaris* (Hildenborough) total RNA probed with dig-labeled *fur*, *rbr*, and *rdl*. Lanes: 1, *D. vulgaris* DNA; 2, dig-labeled RNA molecular size markers; 3, *D. vulgaris* total RNA. Positions of the 1.3-kb band (<) and 0.8-kb band (*) in the *D. vulgaris* total RNA lanes are indicated.

region of *fur*. However, no consensus promoter sequence has yet emerged from the known *D. vulgaris* gene sequences (48, 51, 54). Therefore, based on our present results, the 0.8-kb band could represent either an independent *rbr-rdl* transcript or a processed (or degraded) version of the *fur-rbr-rdl* transcript.

The *ngr* gene, transcript, and gene product, Ngr. The properties of Rdl reported above are clearly distinct from those of Ngr (33). For example, Ngr was reported to be a homodimer of about 54 kDa, to contain a diiron-oxo as well as an FeS_4 site, and to have an N-terminal amino acid sequence different from that found for Rdl. We therefore searched for the Ngr gene by Southern blotting using a degenerate oligonucleotide probe based on the published N-terminal amino acid sequence of Ngr (33). We found that this probe did not hybridize to the 7-kbp *Pst*I fragment of *D. vulgaris* chromosomal DNA in pBP96-4, which contains *fur*, *rbr*, and *rdl* (34). However, the probe did hybridize to a 2.8-kbp *Sal*I/*Pst*I restriction fragment of *D. vulgaris* chromosomal DNA, which, upon sequencing, yielded an ORF encoding a 202-residue protein, whose N-terminal sequence exactly matches that published for Ngr (33) (Fig. 6 and 7). This ORF, designated *ngr*, is preceded by a ribosome binding site (AGGAG) 14 to 10 nucleotides upstream of the start codon and is followed by a putative stem-loop structure starting 50 bp downstream of the *ngr* stop codon (Fig. 6), which possibly signals transcription termination. The amino acid sequence of Ngr shows 33% identity (52% similarity) to that of Rr (Fig. 7) and, consistent with the higher molecular weight previously determined for Ngr (33), contains 11 more residues than Rr. All of the residues which have been identified as iron ligands in *D. vulgaris* Rr (10) are conserved in Ngr. His 56 in Rr, which is near but not coordinated to iron in the crystal structure, is also conserved in Ngr.

An alternate interpretation of the ORF containing *ngr* would add 19 residues onto the N terminus of Ngr in the sequence MGWSTSPPLRPHKTKERCT. A potential ribosome binding site (GAAGG) occurs 9 to 13 nucleotides upstream of this alternate start site. The 19-residue sequence does not resemble any known signal peptide sequence in *Desulfovibrio* species (54), and based on their isolation procedure, Pierik et al. (33) inferred a soluble, cytoplasmic localization for Ngr in *D. vulgaris*. Also, as stated above, the N-terminal amino acid sequence reported for Ngr (33) agrees with that of the shorter 202-residue sequence shown in Fig. 7. Therefore, the significance of this potential N-terminal extension of Ngr is unclear.

Using the shorter 202-residue sequence, we overexpressed *ngr* and then isolated and purified recombinant *D. vulgaris* Ngr from *E. coli* BL21(DE3). The resulting protein had UV/vis absorption, EPR, and gel filtration properties (data not shown) which were not significantly different from those reported for Ngr isolated from *D. vulgaris* (33). The recombinant Ngr showed a ferroxidase specific activity which was approximately 20% of that shown by recombinant Rr (5).

Northern analysis of *D. vulgaris* RNA with *ngr* as a probe revealed a single band at 0.8 kb (Fig. 8). This band is approximately 140 bp longer than that from the putative ribosome binding site upstream of *ngr* to the beginning of the putative stem-loop structure downstream of *ngr* (659 bp) (Fig. 6). Thus, while we have not identified a sequence which is highly homologous to the *E. coli* σ^{70} RNA polymerase promoter upstream of *ngr*, the size of the 0.8-kb Northern band is consistent with a transcription start site within approximately 140 bp upstream of the *ngr* start codon (or within approximately 80 bp upstream of the alternate start site mentioned above). The size of this band is also consistent with the apparent absence of nearby ORFs running in the same direction as *ngr* (Fig. 6). A partial ORF downstream of and running in the opposite direction to *ngr* encodes a protein with strong sequence homology to sev-

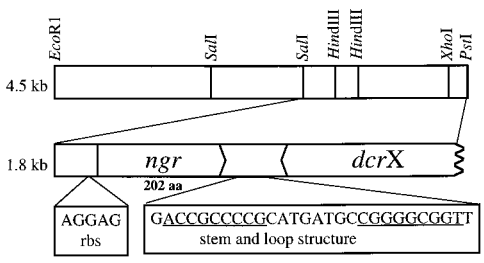

FIG. 6. Restriction map of the 4.5-kbp *Eco*RI/*Pst*I fragment of *D. vulgaris* (Hildenborough) chromosomal DNA containing *ngr*. The 1.8-kbp *Sal*I/*Pst*I fragment (shaded) is expanded to show the locations and relative sizes of *ngr*, which encodes Ngr (aa, amino acid residues), its putative ribosome binding site (rbs), the partial ORF containing the *dcr* homolog, and the intervening putative stem-loop structure.

```
Ngr     1 MKVRAQVPTVKNATNFNMVADSKTAVGSTLENLKAAIAGETGAHAKYTAF 50
Rr      1 ..............MKSLKGSRTE.....KNILTAFAGESQARNRYNYF 30
Ngr    51 AKAAREQGYEQIARLFEATAAAELIHIGLEYALVA....EMEPGYEKPTV 96
Rr     31 GGQAKKDGFVQISDIFAETADQEREHAKRLFKFLEGGDLEIVAAFPAGII 80
Ngr    97 AAPSAYSCDLNLISGANGEIYETSDMYPAFIREAQGEGNSKAVHVFTRAK 146
Rr     81 ADTHA.....NLIASAAGEHHEYTEMYPSFARIAREEGYEEIARVFASIA 125
Ngr   147 LAESVHAERYLAAYNDIDAP......DDDKFHLCPICGYIHKGEDF.EKC 189
Rr    126 VAEEFHEKRFLDFARNIKEGRVFLREQATKWR.CRNCGYVHEGTGAPELC 174
Ngr   190 PICFRPKDTFTAY.... 202
Rr    175 PACAHPKAHFELLGINW 191
```

FIG. 7. Alignment of *D. vulgaris* Ngr and Rr amino acid sequences, using GAP. Residues which have been identified as iron ligands in Rr are starred. + indicates a conserved histidine residue which is near but not ligated to iron in the crystal structure of Rr (10).

eral methyl-accepting chemotaxis proteins from *D. vulgaris* (Hildenborough), the known genes for which have been designated *dcrA* to *dcrL* (12, 53). Consistent with its presumed function as a transcription termination signal, the putative stem-loop structure lies between *ngr* and the *dcr* homolog (Fig. 6).

DISCUSSION

This work establishes that the *D. vulgaris* Rr gene, *rbr*, is cotranscribed with two other genes, one encoding a Fur-like protein and the other encoding a rubredoxin-like protein. No Fur or Fur-like proteins have been previously found in *Desulfovibrio* species or, to our knowledge, in any other strictly anaerobic bacteria. In facultative aerobic bacteria, Fur acts as a global transcriptional regulator of genes involved in iron uptake, apparently using Fe^{2+} as corepressor (29, 47). More recently, Fur has been shown to regulate both positively and negatively several other genes, including those for iron and manganese SODs, toxin production, and acid tolerance (15, 17, 35, 52). Despite the large number of iron proteins found in *Desulfovibrio* species, essentially nothing is known about iron uptake, transport, or storage in sulfate-reducing bacteria. Based on the close resemblance of the protein backbone fold of Rr to those of the iron storage proteins ferritin and bacterioferritin (10), the presence of nonheme iron sites in Rr, and the cotranscription of *rbr* with a *fur*-like gene, a function for Rr in iron homeostasis is indicated. One possibility, given the cotranscription of *fur* and *rbr*, is that the diiron site in Rr participates in iron exchange with Fur, thereby modulating the transcriptional regulatory activity of the latter protein, perhaps while serving as a redox sensor via the FeS_4 site.

Alternative possibilities arise from the fact that the type of diiron site and surrounding protein fold of Rr are characteristic of enzymes which use O_2 as a substrate (10, 20, 30). Although classified as anaerobes, several *Desulfovibrio* strains have been reported to metabolize O_2 in sulfide-free cultures under low partial pressures of O_2 (9, 22, 26). *D. vulgaris* contains a catalase and an SOD (18) and can survive exposure to air for limited periods (8). Under conditions of oxidative stress, the ferroxidase activity of Rr (5) could be one component of a set of enzymes which detoxifies the reaction products of Fe(II) and O_2. However, the relatively low turnovers measured for the ferroxidase activities of Rr and Ngr in vitro and their apparent cytoplasmic localizations suggest that the ferroxidase activities of these proteins by themselves may not be major contributors to [Fe(II) + O_2] sensing/scavenging in vivo. The same characterization probably applies to their apparent SOD activities. Our preliminary results indicate that the SOD specific activities for *D. vulgaris* Rr and Ngr are about the same as for *C. perfringens* Rr (23, 43), and these activities are all 1 to 2 orders of magnitude lower than that reported for the iron SOD isolated from *Desulfovibrio desulfuricans* (18).

A small (14-kDa) nonheme iron protein called desulfoferrodoxin has been found in some sulfate-reducing bacteria (11). Although no sequence homology to Rr or Ngr is evident, desulfoferrodoxin contains an FeS_4 site and a second mononuclear iron site with no more than one cysteine ligand. In *D. vulgaris*, desulfoferrodoxin is the product of the *rbo* gene (28). Recently, expression of the *D. vulgaris rbo* gene product was shown to protect *E. coli sod* mutants against oxidative stress, even though desulfoferrodoxin showed little or no SOD activity in vitro (32). Oxidative destruction of [4Fe-4S] clusters in metabolically crucial enzymes and Fenton chemistry resulting from the subsequently released iron may constitute the primary toxic effects of superoxide (13). Pianzzola et al. (32) proposed that desulfoferrodoxin's protection against oxidative stress could be due to its reduction of oxidized, inactivated [4Fe-4S] clusters, thereby reversing loss of enzyme activity and preventing release of iron. We suggest a somewhat different possibility for Ngr and Rr: these proteins may protect cells against oxidative stress by both scavenging iron from and donating iron to oxidatively damaged [4Fe-4S] proteins. Such a function is consistent with the rescue by *C. perfringens* Rr of oxidatively stressed *E. coli sod* mutants (23, 32) and with the linkage of Rr to iron homeostasis and iron storage proteins discussed above.

Our results show that *D. vulgaris* Rdl has properties similar to those of rubredoxins but is somewhat larger, has six rather than four cysteine residues, and has altered sequence spacing of one of the four cysteine ligand residues. Rubredoxins in anaerobic bacteria are usually assumed to be involved in electron transfer (22, 44). If this is the case for Rdl, then, given its cotranscription, a logical function would be as a redox partner

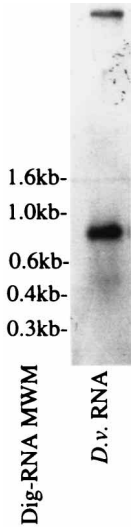

FIG. 8. Northern analysis of *D. vulgaris* (Hildenborough) (*D.v.*) total RNA probed with dig-labeled *ngr*. MWM, molecular size markers.

to Rr. Thus, Rdl together with the FeS_4 site in Rr would seem to imply a redox-linked function for these two proteins.

Our identification and sequencing of *ngr* together with previous results (33) establish that *D. vulgaris* Ngr is very similar to Rr from the same organism. However, despite this resemblance, our results show that *ngr* can be no closer than about 7 kb to *rbr* on the *D. vulgaris* chromosome, and unlike Rr, Ngr is not transcriptionally associated with other proteins. The conservation in one organism of two very similar proteins whose genetic and transcriptional environments differ from each other suggests that these two proteins could perform similar functions on two different sets of cellular components.

In addition to the *C. perfringens* Rr, an *rbr*-like ORF has recently been found in the genome of the methanogenic archaeon *Methanococcus jannaschii* (7). The diversity of microorganisms found to contain *rbr*-like genes and the presence of two such genes in *D. vulgaris* suggest an important, generalized function for Rr-like proteins in anaerobes. The results described here point to a role in iron metabolism, perhaps connected to oxidative stress.

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