

Rubrerythrin and Rubredoxin Oxidoreductase in *Desulfovibrio vulgaris*: a Novel Oxidative Stress Protection System

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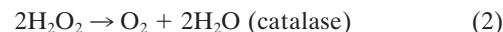
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Evidence is presented for an alternative to the superoxide dismutase (SOD)-catalase oxidative stress defense system in *Desulfovibrio vulgaris* (strain Hildenborough). This alternative system consists of the nonheme iron proteins, rubrerythrin (Rbr) and rubredoxin oxidoreductase (Rbo), the product of the *rbo* gene (also called desulfoferrodoxin). A Δrbo strain of *D. vulgaris* was found to be more sensitive to internal superoxide exposure than was the wild type. Unlike Rbo, expression of plasmid-borne Rbr failed to restore the aerobic growth of a SOD-deficient strain of *Escherichia coli*. Conversely, plasmid-borne expression of two different Rbrs from *D. vulgaris* increased the viability of a catalase-deficient strain of *E. coli* that had been exposed to hydrogen peroxide whereas Rbo actually decreased the viability. A previously undescribed *D. vulgaris* gene was found to encode a protein having 50% sequence identity to that of *E. coli* Fe-SOD. This gene also encoded an extended N-terminal sequence with high homologies to export signal peptides of periplasmic redox proteins. The SOD activity of *D. vulgaris* is not affected by the absence of Rbo and is concentrated in the periplasmic fraction of cell extracts. These results are consistent with a superoxide reductase rather than SOD activity of Rbo and with a peroxidase activity of Rbr. A joint role for Rbo and Rbr as a novel cytoplasmic oxidative stress protection system in *D. vulgaris* and other anaerobic microorganisms is proposed.

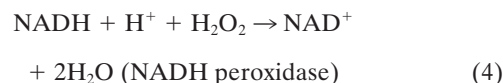
Although sulfate-reducing bacteria are classified as strict anaerobes, many species are aerotolerant and some species have been shown to accumulate at oxic-anoxic interfaces (28). Therefore, sulfate-reducing bacteria must deal with oxidative stress. Some sulfate-reducing bacteria have been shown to contain superoxide dismutases (SODs) and catalases, but other species do not demonstrate the activities expected for these enzymes (15, 18, 43). The apparent absence of SOD and catalase can be rationalized on the basis that their catalytic dismutation reactions (reactions 1 and 2) generate O_2 , which may be disadvantageous for strict anaerobes.

Evidence for an alternative oxidative stress protection mechanism in sulfate-reducing bacteria has begun to emerge. Rubredoxin oxidoreductase (Rbo), which has also been named desulfoferrodoxin (29), is the product of the *rbo* gene in several sulfate-reducing bacteria (7). A *Desulfovibrio vulgaris* strain with the *rbo* gene deleted was shown to be more air sensitive than the wild type (45). The Rbo from *Desulfoarculus baarsii* was shown to complement a *sodA sodB* defect in *Escherichia coli* (31). Rbo has no detectable sequence homologies to known SODs, and little or no SOD activity has been found for Rbos in vitro (26, 35). Nevertheless, the *D. baarsii* Rbo was reported to reduce the steady-state level of intracellular superoxide in the *E. coli sodA sodB* strain (25). This Rbo in vitro showed evidence for superoxide reductase (SOR) activity (26), i.e., reduction of O_2^- , presumably to H_2O_2 , without dismutation (reaction 3).



Rbo is a homodimeric protein, each subunit of which contains two mononuclear nonheme iron centers (11). Center I contains a distorted rubredoxin-type $[Fe(SCys)_4]$ coordination sphere and is presumably an electron transfer center. Center II contains a unique $[Fe(NHis)_4(SCys)]$ site that is rapidly oxidized by O_2^- and is, therefore, the likely site of superoxide reduction (26). The only iron site in a blue nonheme iron protein, nellarodoxin (Nlr) from *Desulfovibrio gigas* (10), closely resembles that of Rbo center II (11).

A second type of nonheme iron protein, rubrerythrin (Rbr), has also been implicated in oxidative stress defense in anaerobic microbes (1, 2, 24). Rbr is a homodimeric protein that contains both a rubredoxin-like $[Fe(SCys)_4]$ center and a non-sulfur, oxo-bridged diiron site (14). The best-characterized Rbr is that from the anaerobic sulfate-reducing bacterium *D. vulgaris* (strain Hildenborough). In vitro, *D. vulgaris* Rbr is able to function as the terminal component of an NADH peroxidase, catalyzing the reduction of hydrogen peroxide to water (reaction 4) (12, 13):



D. vulgaris contains a second Rbr-like protein, called nigrerythrin (Ngr) (27, 32), which also exhibits NADH peroxidase activity (13).

In the present work we present evidence for the roles of Rbo and Rbr in the protection of *D. vulgaris* against exposure to air, superoxide, and hydrogen peroxide. We also describe a *D. vulgaris* gene with high sequence homology to bacterial Fe-

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TABLE 1. Strains and plasmids used in this work

Strain or plasmid	Genotype or relevant characteristic	Source or reference
Strains		
<i>D. vulgaris</i>		
Hildenborough	Wild type, Km ^r	27
L2	Δrbo Cm ^r Km ^r	45
<i>E. coli</i>		
DH5 α	<i>endA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44 thi-1 recA1 gyrA</i> (Nal ^r) <i>relA1</i> Δ (<i>lacIZYA argF</i>) <i>U169 deoR</i> [ϕ 80 <i>dlac(lacZ)</i> M15]	Laboratory stock
QC774	GC4468 Φ (<i>sodA-lacZ</i>)49 Φ (<i>sodB-kan</i>)1- Δ_2 Cm ^r Km ^r	9
NC202	$\Delta lacU169 rpsL$ Φ (<i>katE::lacZ</i> ⁺)131 <i>katG17::Tn10</i>	39
Plasmids		
pCYB1	Cloning/overexpression vector, Ap ^r	New England BioLabs, Inc. (Beverly, Mass.)
pUC18	Cloning vector, Ap ^r	Laboratory stock
pT7-7	Overexpression vector, Ap ^r	40
pDK3-5	pT7-7 derivative with <i>D. vulgaris rbr</i> gene in the <i>NdeI/BamHI</i> sites	17
pNS1	pT7-7 derivative with <i>D. vulgaris ngr</i> gene in the <i>NdeI/BamHI</i> sites	27
pNLR119	pUC18 derivative with a 2.2-kbp <i>EcoRI/HindIII</i> fragment of <i>D. gigas</i> chromosomal DNA containing the <i>nlr</i> gene	This work
pRbr	pCYB1 derivative with <i>D. vulgaris rbr</i> gene in the <i>NdeI/BamHI</i> sites	This work
pRbo	pCYB1 derivative with <i>D. vulgaris rbo</i> gene in the <i>NdeI/SapI</i> sites	This work
pNgr	pCYB1 derivative with <i>D. vulgaris ngr</i> gene in the <i>NdeI/SapI</i> sites	This work
pNlr	pCYB1 derivative with <i>D. gigas nlr</i> gene in the <i>NdeI/HindIII</i> restriction sites	This work

SODs. A joint role for Rbo and Rbr as a novel oxidative stress protection system in anaerobic microorganisms is proposed.

MATERIALS AND METHODS

Molecular biology reagents and procedures. Molecular biology procedures not explicitly described below followed those in references 3 or 36. Restriction enzymes were from either Boehringer Mannheim (Indianapolis, Ind.) or Promega, Inc. (Madison, Wis.).

Bacterial strains and plasmids. The bacterial strains and plasmids used in this work are listed in Table 1.

Media and growth conditions. *D. vulgaris* (Hildenborough) strains were maintained in liquid culture in Postgate's anaerobic medium C (33), supplemented with kanamycin (50 μ g/ml) and chloramphenicol (10 μ g/ml) when appropriate. Agar plates were poured aerobically using Postgate's medium E (33). After 24 h, the plates were transferred to a Coy anaerobic chamber (Coy Laboratory Products, Inc., Grass Lake, Mich.) containing an atmosphere of 5% hydrogen, 10% carbon dioxide, and 85% nitrogen and were allowed to equilibrate for 4 days prior to use. *E. coli* strains were grown aerobically at 37°C in Luria-Bertani (LB), M9, or M63 minimal medium containing ampicillin (100 μ g per ml) when needed for plasmid maintenance. Where noted, M63 minimal medium was supplemented with 0.1% Casamino Acids and 0.5 mg of vitamin B1 per liter.

Cell extracts. To prepare periplasmic and cytoplasmic fractions of *D. vulgaris* cells, 1-liter anaerobic cultures were grown overnight at 37°C to early stationary phase. All further workup was done under an aerobic atmosphere. Periplasmic and cytoplasmic fractions were prepared essentially as described previously (32, 42). The cells were harvested by centrifugation for 20 min at 4,000 \times g and washed once with 50 mM Tris-HCl (pH 8.0). The cells were resuspended in 10 ml of 50 mM Tris-HCl-50 mM EDTA (pH 9.0) and were incubated on ice, with occasional stirring, for 45 min. After centrifugation, the red-colored supernatant, designated the periplasmic fraction, was removed. Cytochrome *c*₃, a periplasmic protein, was measured by absorbance at 410 nm for oxidized samples and at 419 nm for dithionite reduced samples. The pellet was resuspended in 10 ml of 50 mM Tris-HCl-50 mM EDTA (pH 9.0) and was lysed to release the cytoplasmic fraction by three passes through a French pressure minicell at 18,000 psi. Desulfoviridin, a cytoplasmic protein, was detected in cytochrome fractions spectrophotometrically, as determined by $A_{628} - (A_{608} + A_{648})/2$ (21). The periplasmic and cytoplasmic fractions were dialyzed against 50 mM phosphate buffer (pH 7.8). Protein quantification was performed by the Bio-Rad DC protein assay using bovine serum albumin standards.

Superoxide dismutase activity. A standard assay that relies on the inhibition of cytochrome *c* reduction by superoxide generated from xanthine/xanthine oxidase

was used (6). Aliquots (~100 μ l) of cytoplasmic and periplasmic fractions were added to achieve ca. 50% inhibition of cytochrome *c* under standard assay conditions.

***D. vulgaris* oxidative stress assays.** For exposure to O₂ and superoxide, 100-ml cultures of *D. vulgaris* wild type and strain L2 were grown in anaerobic medium C at 37°C until mid-log phase (optical density at 600 nm [OD₆₀₀], ~0.6). Forty-five-milliliter aliquots of the anaerobic cultures were transferred to sterile 250-ml flasks and continuously stirred with a magnetic stirrer in air at room temperature. For exposure to superoxide, paraquat (methyl viologen) (PQ) was added to a concentration of 10 μ M. Aliquots (0.1 to 1 ml) of these air-exposed cultures were removed hourly, transferred to the anaerobic chamber, diluted (10⁹ to 10⁶) into anaerobic medium C, and immediately plated onto medium E plates in triplicate. After incubating the cultures for 5 days at 37°C, colonies were counted and the number of surviving CFU/ml was determined. As noted previously (45), the oxidatively stressed *D. vulgaris* cells produced variable sized colonies, making them difficult to enumerate. For the experiments reported in the present work, all colonies (large and small) were enumerated after 5 days of anaerobic incubation, and each reported CFU/ml number is the average from three plates.

To test for hydrogen peroxide sensitivity, anaerobic mid-log-phase cultures were spread onto medium E plates. Hydrogen peroxide (2.5 or 5.0 μ mol in 10 μ l of final volume) was spotted onto circular glass-fiber membranes placed on the cell lawns. The plates were incubated anaerobically for 3 days, and the diameters of growth inhibition surrounding the filters were measured to compare sensitivities.

Cloning of *rbo*, *rbr*, *ngr*, and *nlr*. The *D. vulgaris* genes encoding Rbr, Ngr, and Rbo and the *D. gigas* gene encoding Nlr were amplified by PCR and cloned into the overexpression vector pCYB1. *rbr* and *ngr* were amplified from template plasmids pDK3-5 and pNS1, respectively. *rbo* was amplified from *D. vulgaris* chromosomal DNA. *D. gigas nlr* was cloned from *D. gigas* chromosomal DNA using a procedure similar to that described by Silva et al. (38), and pNLR119 (Table 1) was used as the template. Both *D. vulgaris* and *D. gigas* chromosomal DNAs had been isolated as previously described (27). Sequences of the oligonucleotide primers duplicated the N- and complementary C-terminal 14 bases of each gene using sequences from GenBank accession numbers M28848 (*rbo*), U82323 (*rbr*), U71215 (*ngr*), and AF034965 (*nlr*). An *NdeI* restriction site was incorporated into the start codon of each N-terminal primer. A stop codon (TGA) and an appropriate restriction site, either a *SapI* (*rbo*, *ngr*), *BamHI* (*rbr*), or *HindIII* (*nlr*) site, were added to the 3' end of each C-terminal primer. All PCRs were carried out in 50- μ l volumes in 1 \times PCR buffer (Boehringer Mannheim) containing 1.5 mM MgCl₂, 200 mM deoxynucleoside triphosphate, 0.05 mM primers, and 2.5 U of *Taq* DNA polymerase (Promega). The amplification procedure consisted of a hot start of the template DNA and primers at 94°C for

10 min, followed by 30 cycles of 94°C for 1 min, 60°C for 1 min, and after addition of the remaining reaction components, 72°C for 2 min. The purified PCR products were digested with the appropriate restriction enzymes and ligated into corresponding restriction sites of pCYB1. Nucleotide sequences of the genes in the recombinant plasmids were verified by DNA sequencing, which was carried out in the Molecular Genetics Instrumentation Facility at the University of Georgia.

***E. coli* H₂O₂ survival assay.** All steps were carried out aerobically. Cultures of *E. coli* strain NC202 (*katE katG*) harboring the pCYB1-based vectors listed in Table 1 were grown at 37°C to an OD₆₀₀ of 1.0 in LB-ampicillin medium. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to 2 mM and the culture was allowed to grow to an OD₆₀₀ of 2.2. Aliquots of the cultures were diluted into 50 ml of LB medium in a 250-ml flask without antibiotics to an initial OD₆₀₀ of 0.2. Hydrogen peroxide from a 30% aqueous stock was added to achieve a concentration of 2.5 mM H₂O₂. Aliquots of the cultures were removed immediately before and 30 min after the H₂O₂ challenge. These aliquots were diluted in LB medium containing catalase (100 μg/ml) and plated in triplicate on LB agar plates containing catalase (100 μg/ml). Colonies were enumerated after 3 or 4 days at 37°C.

SOD complementation assay. *E. coli* QC774 (*sodA sodB*) cultures harboring one of the pCYB1-derived expression vectors were grown aerobically to saturation in M63-ampicillin medium supplemented with amino acids and vitamin B1 and were diluted to an initial OD₆₀₀ of ca. 0.06 in aerobic M63-ampicillin medium without supplements. The cultures were incubated with shaking at 37°C, and the growth rate was monitored by measuring the OD₆₀₀.

Cloning of a *D. vulgaris* SOD gene. A conserved amino-acid-sequence region found in several bacterial Fe-SODs, DVWEHAYYLDYQN, was used to design a degenerate oligonucleotide probe with the sequence 5'-GAYGTNTGGGARCAYGNTAYTAYCTNGAYTAYCARAAAY-3'. The 3' end of the probe was labeled with digoxigenin-ddUTP and then used in Southern blotting of *D. vulgaris* (Hildenborough) chromosomal DNA, both according to the protocol for the Genius system (Boehringer Mannheim). The probe hybridized to a 4.5-kbp *EcoRI/HindIII* restriction fragment of *D. vulgaris* chromosomal DNA. Therefore, *EcoRI/HindIII* restricted DNA fragments in the 4- to 5-kb range were isolated from an 0.8% low melting agarose gel by electroelution and were ligated into pUC18. The ligation mixture was transformed into *E. coli* DH5α and plated onto M9-ampicillin agar plates containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) at 2 mg/ml. White colonies were selected to inoculate 1.5-ml cultures grown in LB-ampicillin. Plasmids were purified by alkaline lysis from six combined cultures and were screened for the presence of the *sod* gene by Southern hybridization. Screening of 100 plasmid pools resulted in the isolation of a plasmid containing a 4.5-kbp *EcoRI/HindIII* fragment containing the *D. vulgaris sod*. Sequencing of the cloned gene was performed at the Molecular Genetics Instrumentation Facility at the University of Georgia.

Nucleotide sequence accession number. The newly determined sequence was deposited in the GenBank database under accession no. AF034841.

RESULTS

Rbo protects *D. vulgaris* from exposure to superoxide but not to hydrogen peroxide. Survival of the *D. vulgaris* Δ*rbo* strain, L2, was previously shown to be lower than that of the wild type when anaerobically growing cells were diluted into aerobic medium and then exposed to air for several hours (45). PQ can be used to generate a steady-state level of superoxide within bacterial cells under aerobic conditions. We therefore compared the survival of *D. vulgaris* wild type with that of strain L2 after exposure to air + 10 μM PQ ([air + PQ]). The viability of log-phase *D. vulgaris* cells, as surviving CFU/ml exposed to [air + PQ] over a time course of 4 h, is shown in Fig. 1A, normalized to the air-only surviving CFU/ml. The unnormalized data are plotted in Fig. 1B. For cells of both the wild type and strain L2 that were exposed to air only, we observed a steeper drop-off in cell survival versus time than observed previously (45). This phenomenon can be attributed to the larger surface area for aeration used in our experiments. As can be seen in Fig. 1B, at 4 h exposure to air only, the surviving CFU/ml for strain L2 ($1.03 \pm 0.04 \times 10^8$) was greater than that

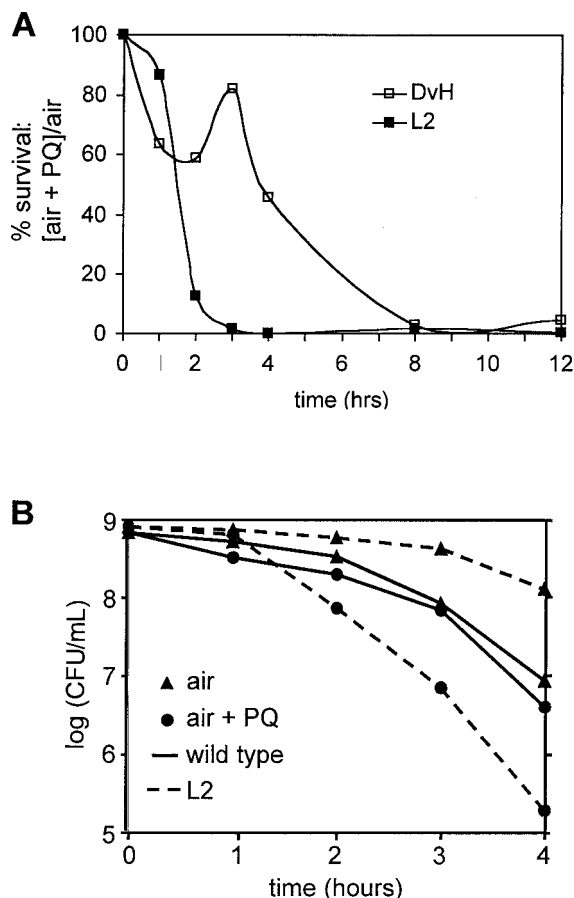


FIG. 1. Viabilities of *D. vulgaris* wild type and L2 strains following exposure to either air or air plus 10 μM PQ. (A) Surviving CFU versus the time of the [air+PQ]-exposed cells normalized to the surviving CFU of the air-only exposed cells. (B) The actual CFU/ml versus the time for both air only- and [air + PQ]-exposed cells.

of wild type ($9 \pm 4 \times 10^6$). This artifact could be due to the greater initial viable cell density we used for strain L2 than for wild type, thereby affording greater protection against oxidative stress at the earlier exposure times. By 8 h of air-only exposure, the surviving CFU/ml of strain L2 ($9 \pm 1 \times 10^4$) had decreased below that of wild type ($2.1 \pm 0.1 \times 10^5$), consistent with the greater air sensitivity of strain L2 previously observed (45). Strain L2 continued to exhibit lower cell survival at 12 h of air-only exposure (data not shown). By 24 h of air-only exposure neither the wild type nor strain L2 showed any survival under our conditions.

The novel observation in the present work occurred upon the addition of 10 μM PQ to the air-exposed *D. vulgaris* cells. Both the wild type and strain L2 lost viability faster when exposed to [air + PQ] than to air only but the effect of PQ was much greater on strain L2 (Fig. 1A). After 4 h the survival of wild-type cells exposed to [air + PQ] had decreased by a factor of ~2 relative to those exposed to air only ($9 \pm 4 \times 10^5$ versus $4 \pm 2 \times 10^6$), whereas the survival of the L2 [air + PQ]-exposed cells decreased by a factor of ~500 ($1.03 \pm 0.04 \times 10^8$ versus $1.9 \pm 0.2 \times 10^5$) relative to the cells exposed to air only. The much greater [air + PQ] versus air-only sensitivity of

TABLE 2. H₂O₂ sensitivities and SOD activities of *D. vulgaris* (Hildenborough) strains

<i>D. vulgaris</i> strain	H ₂ O ₂ growth inhibition diam (mm) with:		SOD activities (U/mg of protein) ^a	
	2.5 μmol H ₂ O ₂	5.0 μmol H ₂ O ₂	Periplasm	Cytoplasm
Wild type	3.00 ± 0.10	3.50 ± 0.10	5.1 ± 0.1	1.1 ± 0.1
L2 (<i>Δrbo</i>)	2.90 ± 0.10	3.70 ± 0.10	5.4 ± 0.1	1.3 ± 0.1

^a Subcellular fractions prepared as described in Materials and Methods.

strain L2 compared to wild type was verified in two other experiments (data not shown). The addition of up to 100 μM PQ did not affect the anaerobic growth (measured as OD₆₀₀) of either *D. vulgaris* wild type or L2 cultures (data not shown).

Anaerobic peroxide sensitivity was tested by growth inhibition on agar plates containing lawns of either wild type or L2 and filter disks saturated with hydrogen peroxide solutions. We found no significant difference in the zones of growth inhibition between wild type and L2 using two different amounts of hydrogen peroxide (Table 2).

SOD activity of *D. vulgaris* is not affected by the absence of Rbo and is concentrated in the periplasmic fraction. A low level of SOD activity had previously been reported in *D. vulgaris* cell extracts (18). In order to localize and quantitate this activity we tested periplasmic and cytoplasmic fractions of *D. vulgaris* wild type and strain L2 for SOD activity using a standard assay (Table 2). The fidelity of periplasmic and cytoplasmic preparations were checked by comparing cytochrome *c*₃ (a periplasmic protein) with desulfovirdin (a cytoplasmic protein) levels using a previously described spectrophotometric method (32, 42). No desulfovirdin could be detected in the periplasmic fractions, while more than 70% of the total cytochrome *c*₃ detected was seen in the periplasmic fractions (data not shown). The specific SOD activities (per milligram of total protein) of these subcellular fractions were unaffected by the absence of the *rbo* gene. For both strains, the specific SOD activity was approximately five times greater in the periplasmic

fractions than in the cytoplasmic fractions (Table 2), consistent with the presence of a periplasmic SOD in *D. vulgaris*.

***D. vulgaris* has an Fe or Mn SOD-like gene.** Using a degenerate oligonucleotide probe that was designed to encode a conserved region of Fe-SOD sequences, we cloned a *D. vulgaris* gene encoding a protein with 50% sequence identity (59% similarity) to that of *E. coli* Fe-SOD, when aligned as shown in Fig. 2. The N terminus for the *D. vulgaris* SOD was taken as the first methionine that codes for a continuous open reading frame and has a recognizable ribosome binding site (AGGAG) 6 bp upstream. Analysis of the amino acid sequence using SignalP (30) suggested the existence of a 34-residue export leader peptide with the most likely cleavage site between residues A34 and A35. As shown by the sequence alignments in Fig. 3, the putative leader peptide of the *D. vulgaris* SOD resembles those that are commonly used for export of bacterial redox proteins (5) with the notable exception that one of the two residues of a conserved arginine pair is replaced by a serine. We have independently confirmed this replacement by examination of the preliminary genome sequence for *D. vulgaris* (Hildenborough) (The Institute for Genomic Research, personal communication). The implication is that the *D. vulgaris* Fe- or Mn-SOD is periplasmic, in contrast to the Fe-SOD from *E. coli*, which is known to be cytoplasmic. Our database searches have not located a complete amino acid or nucleotide sequence of a recognizable SOD from any other sulfate-reducing bacterium. None of our attempts to express this *D. vulgaris* SOD gene in *E. coli*, including QC774, have resulted in an active SOD.

Rbo but not Rbr or Nlr can complement *E. coli* *sodA* *sodB*. *E. coli* strain QC774 is deficient in both *sodA* and *sodB* encoding the Mn and Fe SODs, respectively (9). Under aerobic conditions this strain is unable to grow in minimal medium lacking amino acid supplements. This phenotype is attributed to superoxide damage to iron-sulfur enzymes in the biosynthetic pathway of branched chain amino acids and to Fenton chemistry resulting from increased free iron levels (41). Figure 4 shows that plasmid-borne expression of *D. vulgaris* Rbo is able

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Dv 1 MPCNVTRSSFMGLCAAAVVTLGVPFFGARVAHAAGEAEA 40
      +
Ec  -----MS 2

Dv 41 FPMPLPYPDTALEPAISARTISFHYGKHTAAYANLNKAVAGTPMATMRLLEDVVKLVAG
      F +P LPY AL P ISA TI +HYGKH Y NLN + GT LE++++
Ec 183 FELPALPYAKDALAPHISAETIEYHYGKHHQTYVTNLNLIKGTAFEGKSLEEIIIR----
      *

Dv 101 DPAKGVLFNNAHLWNHTFYWAGMKPGGGTTPPAKVADALKASFGSVEACMEQLSEAAKT
      ++G +FNNA +WNHTFYW + P GG P KVA+A+ ASFGS Q ++AA
Ec 351 -SSEGGVFNNAQVWNHTFYWNCLAPNAGGEPTGKVAEAIASFGSFAFDKQAQFTDAAIK
      *

Dv 161 QFASGWAWLAKGTENGKPVLVKVMKTGNADNPMTQGLTPVLTIDVWEHAYYLDYQNKRPDY
      F SCW WL K ++ GK L ++ T NA P+T TP+LT+DVWEHAYY+DY+N RP Y
Ec 528 NFGSGWTWLKNSD-GK--LAIVSTSNAGTPLTTDATPLLTVDVWEHAYYIDYRNARPGY
      * *

Dv 221 VKAFFEKLWNWDEVATRL 238
      ++ F+ LVNW+ VA L
Ec 699 LEHFW-ALVNWEFVAKNL 749

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FIG. 2. Amino acid sequence alignment of the putative *sod* gene product from *D. vulgaris* with the Fe-containing SOD from *E. coli* (GenBank accession no. J03511.1). The putative leader sequence cleavage site in the *D. vulgaris* SOD sequence is shown in italics between residues A34 and A35. Asterisks (*) indicate iron ligand residues in *E. coli* SOD (22). +, amino acids are similar to each other.

Db_HysB	M	SLSRREFVKL	CSAGVAGLGI	SQIYHPGIVH	A
Ws_HydA	MLEEK	GIERRDFMKW	AGAMTAMLSL	PATFTPLTAK	A
Sa_SoxF		MDRRTFRL	YLLVGAATAV	APVIKPALDY	VGY
Ec_HybA		MNRRNFIKA	ASCGALLTGA	LPSVSHA	
DvH_Sod	MPC	NVTRSSFMLG	CA.AAAVVTL	GVPFFGARVA	HA
Ec_NrfC	M	TWSRRQFLTG	VGVLAAVSGT	AGRVVA	
Hi_NrfC	MT	VCRRNFVSG	MGAVILMTGT	SLPAFA	
Sm_NosZ	MSNEETKM	RLNRRQMLGT	TAFMAAAGAV	GAGGALTLSG	GTATPARA
P_s_NosZ	MSDKDS	KNTPQVPEKL	GLSRRGFLGA	SAVTGAAVAA	TALGGAVMTR
				ESWA	
Consensus		SRRxFLK			

FIG. 3. Sequence alignment of export signal peptides from selected bacterial and archaeal redox proteins with the putative signal peptide of the *D. vulgaris* (Hildenborough) SOD (DvH_SOD). Alignment of the sequences follows that in reference 5. Sequence sources (GenBank accession numbers) are as follows: Db_HysB, *Desulfomicrobium baculatum* NiFeSe hydrogenase (P13063); Ws_HydA, *Wolinella succinogenes* NiFe hydrogenase (S33852); Sa_SoxF, *Sulfolobus acidocaldarius* Rieske iron-sulfur protein (S56156); Ec_HybA, *E. coli* hydrogenase (P37179); Ec_NrfC, *E. coli* putative iron-sulfur protein (P32708); Hi_NrfC, *Haemophilus influenzae* putative iron-sulfur protein (P45015); Sm_NosZ, *Sinorhizobium meliloti* nitrous oxide reductase (Q59746); and P_s_NosZ, *Pseudomonas stutzeri* nitrous oxide reductase (P19573).

to restore aerobic growth to this strain whereas expression of *D. vulgaris* Rbr and Ngr and *D. gigas* Nlr are not. Strain QC774 will grow aerobically in rich medium (LB) but unlike the wild type, this growth is inhibited by 10 μM PQ. Rbo expression completely restored growth to 10 μM PQ-stressed QC774 in LB, whereas once again expression of Rbr, Ngr, or Nlr did not (data not shown). Preinduction of protein overexpression by the addition of IPTG to 0.4 mM did not change these outcomes. The plasmid-expressed Rbr, Rbo, and Nlr have all been isolated from QC774 (E. D. Coulter, N. V. Shenvi, and D. M. Kurtz, Jr., unpublished data), demonstrating that these proteins can be expressed in this strain.

***D. vulgaris* Rbr and Ngr but not Rbo are able to rescue *E. coli* kat from H₂O₂ stress.** *E. coli* strain NC202 lacks both *katG* and *katE* genes encoding catalase/peroxidase (HPI) and catalase (HPII), respectively (39). Plasmid-borne expression of *D. vulgaris* Rbr or Ngr increased the survival of this strain when exposed to H₂O₂ under aerobic conditions, as shown in Fig. 5. H₂O₂-exposed NC202 cells expressing Rbr showed at least 25% higher viability than those containing the parent plasmid, and cells expressing Ngr were fully protected against H₂O₂

exposure. Expression of Rbo failed to provide any protection to the H₂O₂-exposed NC202 strain and in fact reduced its viability to a few percent upon peroxide stress. Overexpression of these heterologous proteins in NC202 was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown).

DISCUSSION

The results reported here indicate that *D. vulgaris* Rbo functions as an intracellular superoxide scavenger but not as an SOD, that *D. vulgaris* Rbr and Ngr protect against hydrogen peroxide stress in vivo, and that *D. vulgaris* contains a periplasmic Fe- (or possibly Mn-)SOD.

Sequence analysis of the *D. vulgaris* Fe-SOD gene indicated the presence of an export leader peptide commonly found in periplasmic redox proteins. The replacement of one of the two residues of a highly conserved arginine pair by a serine is unusual if not unique for redox protein leader peptides, including those previously reported in sulfate-reducing bacteria (44). Consistent with the implied periplasmic localization, we found that the majority of the SOD activity in *D. vulgaris* is associated with the periplasmic fraction of cell extracts, even in the *rbo*-deleted strain. We have found no data addressing the localizations of SODs in other sulfate-reducing bacteria. A few

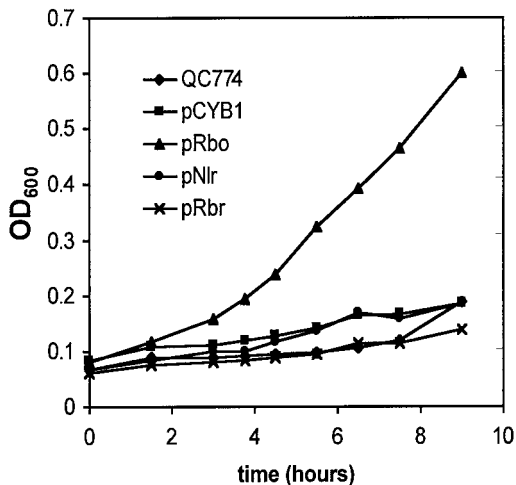


FIG. 4. Aerobic growth versus the time at 37°C in M63 medium of *E. coli* QC774 (*sodA sodB*) containing pCYB1-based plasmids expressing *D. vulgaris* Rbo and Rbr and *D. gigas* Nlr.

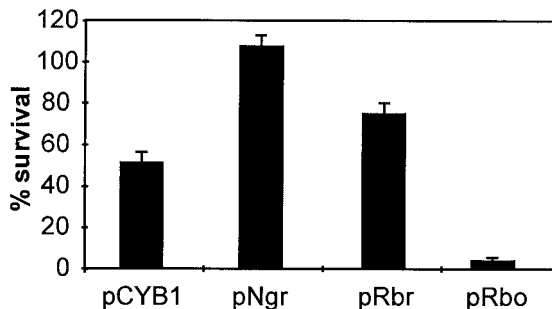


FIG. 5. Viability of aerobically grown *E. coli* NC202 (*katG katE*) expressing plasmid-borne *D. vulgaris* Rbr, Ngr, or Rbo genes following a 30-min exposure to 2.5 mM H₂O₂. IPTG (0.4 mM) was added to induce overexpression of the genes prior to H₂O₂ exposure.

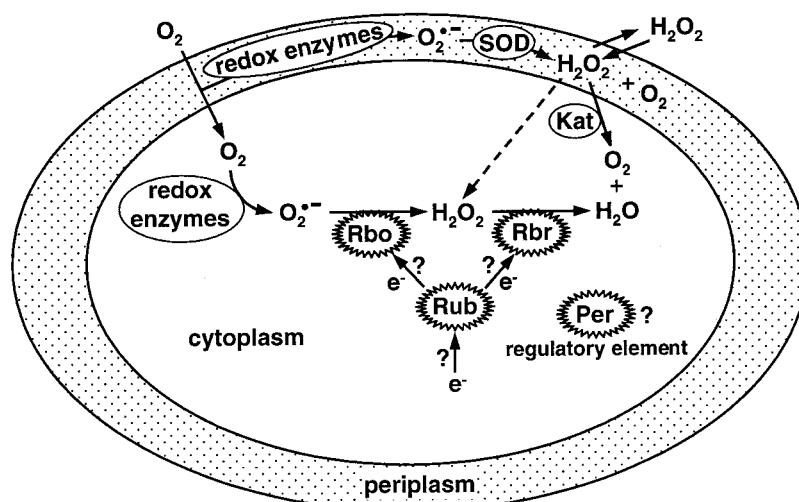


FIG. 6. Proposed model for an Rbo/Rbr oxidative stress protection system in *D. vulgaris*.

other periplasmic Fe/Mn-SODs have been reported, but only from aerobes (4, 16, 37).

A *D. vulgaris* Δrbo strain was previously found to be more air sensitive than wild type (45) but our results show that this air sensitivity is greatly magnified by PQ, a known intracellular superoxide generator. Presumably, the reduction in viability of the Δrbo strain exposed to air only is also due to intracellular generation of superoxide, but at a much slower rate than in the [air + PQ]-exposed cells. The *D. vulgaris* Δrbo strain showed no significant difference in the SOD activity of cellular extracts relative to wild type, indicating that Rbo is not an SOD. We therefore conclude that Rbo protects *D. vulgaris* against internal superoxide stress by functioning as an SOR (reaction 3). The reduction in viability of the aerobically grown *E. coli* catalase-deficient strain upon overexpression of Rbo can be explained by Rbo-catalyzed production of excess hydrogen peroxide from endogenously generated superoxide. A BLAST search of the preliminary *D. vulgaris* genome sequence (The Institute for Genomic Research, personal communication) revealed no other Rbo or Fe-SOD homologs. All available evidence indicates that Rbos are cytoplasmic proteins whereas our results strongly suggest that the *D. vulgaris* SOD is periplasmic. A proposed model for compartmentalized superoxide generation and scavenging in *D. vulgaris* is presented in Fig. 6.

We found that neither *D. vulgaris* Rbr nor Ngr is capable of restoring growth to the *E. coli* *sodA sodB* strain in aerobic minimal medium. Rbr and Ngr are, however, capable of increasing the survival of an *E. coli* catalase-deficient strain that was exposed to hydrogen peroxide. This protection by Rbr and Ngr apparently occurs in competition with at least some of the 30 or more other proteins that are induced in response to peroxide stress in *E. coli* (34). As is the case for Rbo, the protection against hydrogen peroxide by Rbr and Ngr presumably requires an endogenous electron source, perhaps functioning analogously to the *in vitro* NADH peroxidase activity (reaction 4) (12, 13). Rbo and Rbr (and Ngr) may thus be complementary components of an alternative oxidative stress defense in *D. vulgaris*, as shown in Fig. 6.

Heme-containing catalases have been isolated from *D. vulgaris* (Hildenborough) and *D. gigas* (15, 18), and a catalase gene has previously been reported from *D. vulgaris* (Miyazaki F) (GenBank accession no. AB020341). Neither the Miyazaki nor the Hildenborough catalase genes (The Institute for Genomic Research, personal communication) contain a recognizable signal peptide sequence, which indicates that *D. vulgaris* catalase is cytoplasmic, as is Rbr (32). Since heme catalases typically have K_m s for H_2O_2 in the millimolar to molar range (46), the *D. vulgaris* catalase might complement the intracellular Rbr-catalyzed hydrogen peroxide removal by diminishing high concentrations of extracellular hydrogen peroxide, which is freely diffusible across cell membranes (Fig. 6).

Endogenous electron donors to Rbo or Rbr have not been identified either when complementing the *E. coli* strains or in the native organism. The inability of plasmid-expressed *D. gigas* Nlr to restore aerobic growth to *E. coli* *sodA sodB* implies that center I $\{[Fe(SCys)_4]\}$, which is presumably the electron transfer center of Rbo (and not present in Nlr), as well as center II, is required for complementation by Rbo. In *D. vulgaris*, one potential redox partner is rubredoxin, whose gene, *rub*, is cotranscribed with *rbo* (7). A *rub* gene is also adjacent to the *rbo* gene in *D. baarsii* (Fig. 7) (31). Based on genetic proximity, Rbr could also use rubredoxin-like proteins as redox partners. In *D. vulgaris* (Hildenborough), Rbr is cotranscribed with Rdl, yet another class of rubredoxin-type protein (23, 27).

The *rbr* gene in *D. vulgaris* (Hildenborough) is transcribed with a third gene designated *fur* (27), which encodes a protein showing highest sequence homology to PerR, a peroxide-responsive regulatory protein in *Bacillus subtilis* (8, 47). The product of the *fur*-like gene may, therefore, regulate the Rbo-Rbr oxidative stress response in *D. vulgaris*. The genes encoding Rbo and Rbr (and Ngr) occur in separate operons in *D. vulgaris* (7, 27). However, as diagrammed in Fig. 7, in other anaerobic microorganisms Rbo- and Rbr-like genes are located either in succession or within what appears to be the same operon.

To date, Rbo and Rbr (or their genes) have been reported only in anaerobic or microaerophilic bacteria and archaea,

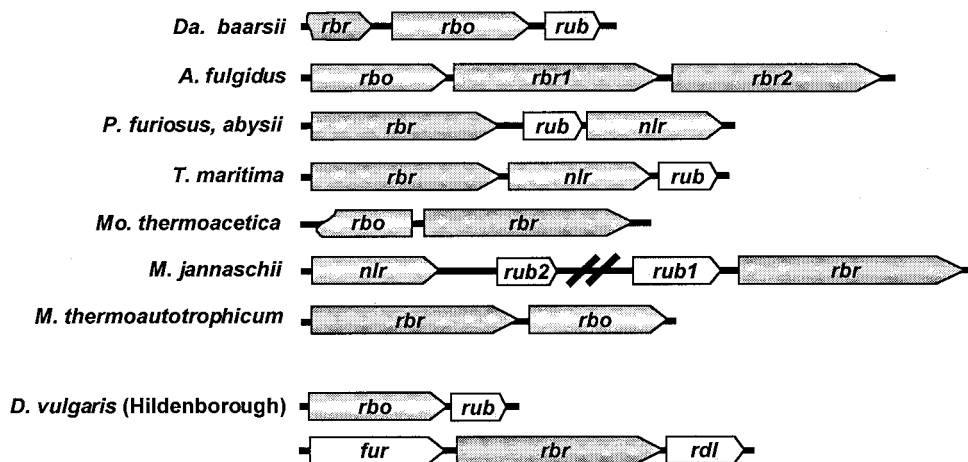


FIG. 7. Diagrams of open reading frames encoding adjacent *rbo*, *rbr*, and *rub* homologs from anaerobic bacteria and archaea. *rub*, rubredoxin gene; *rbo*, Rbo homolog gene; *nlr*, neelaredoxin homolog gene; *rdl*, rubredoxin-like protein gene; *fur*, Fur homolog gene. Horizontal distances between boxes are proportional to the relative spacings of the genes. Arrow-shaped orientations of boxes show putative transcription directions. Open reading frames were derived from sequences deposited in GenBank with the following accession numbers: X99543 (*D. baarsii*), AE001047 (*Archaeoglobus fulgidus*), AF156097 (*P. furiosus*), AJ248285 (*Pyrococcus abyssii*), AE001739 (*Thermotoga maritima*), AF202316 (*Moorella thermoacetica*), U67520 (*Methanococcus jannaschii*), AE000854 (*Methanobacterium thermoautotrophicum*), M28848 [*Desulfovibrio vulgaris* (Hildenborough) *rbo-rub*] and U82323 [*D. vulgaris* (Hildenborough) *fur-rbr-rdl*].

whereas we have found no Rbo or Nlr or Rbr homologs in sequenced genomes of aerobes (as of June 2000). We therefore propose that Rbo-Rbr constitutes an alternative oxidative stress protection system in anaerobes and microaerophiles operating similarly to that diagrammed in Fig. 6. Some aspects of our proposal resemble those recently suggested for oxidative stress protection in the anaerobic hyperthermophilic archaeon *Pyrococcus furiosus* (19). *P. furiosus* contains an Nlr-like protein with superoxide reductase activity as well as an Rbr, the genes for which are tandemly located (Fig. 7). The microorganismic segregation of SOD-catalase between aerobes and anaerobes appears to be less distinct than for Rbo-Rbr (20). The latter segregation suggests that the Rbo-Rbr oxidative stress protection system is well suited to anaerobic life in an aerobic world.

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