

Function of Oxygen Resistance Proteins in the Anaerobic, Sulfate-Reducing Bacterium *Desulfovibrio vulgaris* Hildenborough

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Two mutant strains of *Desulfovibrio vulgaris* Hildenborough lacking either the *sod* gene for periplasmic superoxide dismutase or the *rbr* gene for rubrerythrin, a cytoplasmic hydrogen peroxide (H₂O₂) reductase, were constructed. Their resistance to oxidative stress was compared to that of the wild-type and of a *sor* mutant lacking the gene for the cytoplasmic superoxide reductase. The *sor* mutant was more sensitive to exposure to air or to internally or externally generated superoxide than was the *sod* mutant, which was in turn more sensitive than the wild-type strain. No obvious oxidative stress phenotype was found for the *rbr* mutant, indicating that H₂O₂ resistance may also be conferred by two other *rbr* genes in the *D. vulgaris* genome. Inhibition of Sod activity by azide and H₂O₂, but not by cyanide, indicated it to be an iron-containing Sod. The positions of Fe-Sod and Sor were mapped by two-dimensional gel electrophoresis (2DE). A strong decrease of Sor in continuously aerated cells, indicated by 2DE, may be a critical factor in causing cell death of *D. vulgaris*. Thus, Sor plays a key role in oxygen defense of *D. vulgaris* under fully aerobic conditions, when superoxide is generated mostly in the cytoplasm. Fe-Sod may be more important under microaerophilic conditions, when the periplasm contains oxygen-sensitive, superoxide-producing targets.

Desulfovibrio spp. growing with sulfate or other oxidized sulfur anions as the electron acceptor have a certain tolerance to air exposure. The finding that *Desulfovibrio* spp. can reduce oxygen to water, with hydrogen or organic acids as electron donor, and can couple this process to the production of ATP led to the idea that these organisms also engage in aerobic respiration (5). Oxygen is reduced by a cytoplasmic pathway with rubredoxin:oxygen oxidoreductase (Roo) as the terminal oxidase (7, 30). Because this pathway does not involve a membrane-bound terminal oxidase, it is unclear how ATP synthesis is coupled to oxygen reduction. However, genes for a cytochrome *c* oxidase (Cox) have been found in *Desulfovibrio vulgaris* Miyazaki (14), and a cytochrome *bd* terminal oxygen reductase (Cbd) has been purified from *D. gigas* membranes (18). Genes for both Cox and Cbd are present in the genome sequence of *D. vulgaris* Hildenborough (<http://www.tigr.org>). An electron transport chain involving membrane-bound Cox or Cbd is thus a possibility in *Desulfovibrio* spp. and is more likely to couple proton-motive-force-dependent ATP synthesis to oxygen respiration.

Irrespective of whether oxygen is respired with or without coupled ATP synthesis, its presence and use will give rise to partially reduced, highly reactive oxygen species (ROS). *D. vulgaris* enzymes that detoxify ROS include superoxide reductase (Sor) (3, 13, 24, 34) and rubrerythrin (Rbr), which has NADH-dependent H₂O₂ reductase activity (4, 22, 28). Sor and Rbr reduce superoxide and hydrogen peroxide to water with-

out regeneration of oxygen, a feature that is important for oxygen detoxification in anaerobes (13). These proteins are widely distributed in anaerobes and are sometimes found in a single operon (20). Another enzyme, superoxide dismutase (Sod), which is involved in the elimination of superoxide anions, has been identified in several species of *Desulfovibrio* (6, 11). Recently, the gene encoding Sod has been cloned and sequenced from *D. vulgaris* Hildenborough (20). This protein exhibits 50% sequence identity to Fe-Sod or Mn-Sod from *E. coli*. The N-terminal sequence contains a double-arginine motif characteristic of signal peptides of other bacterial redox proteins, suggesting this Sod to be periplasmic (20) in contrast to Sor, which is cytoplasmic. *D. vulgaris* also contains a cytoplasmic catalase that acts as a second system (in addition to Rbr) to eliminate hydrogen peroxide (20). Thus, *D. vulgaris* contains oxygen defense proteins that are typical of both the aerobic (Sod and Kat) and the anaerobic (Sor and Rbr) microbial world. *D. vulgaris* Hildenborough is one of the few microorganisms that contain both systems, with other examples being *D. gigas* and *Methanothermobacterium thermoautotrophicum* (6, 34, 36). In order to delineate the importance of these defense proteins, we constructed *sod* and *rbr* mutants of *D. vulgaris* Hildenborough. Their properties are compared here with those of the wild-type strain and of an *sor* mutant obtained earlier (42).

MATERIALS AND METHODS

Materials. Restriction and DNA modification enzymes and bacteriophage λ DNA were obtained from Pharmacia. [α -³²P]dCTP (10 mCi/ml; 3,000 Ci/mmol) was from ICN. Mixed gas (85% [vol/vol] N₂, 10% [vol/vol] CO₂, and 5% [vol/vol] H₂) was from Praxair Products, Inc. Anti-mouse and anti-rabbit immunoglobulin G alkaline phosphatase (AP)-linked antibodies were from New England Biolabs.

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TABLE 1. Bacterial strains, primers, vectors, and plasmids used

Strain, plasmid, or primer	Genotype, sequence (position), and/or comment(s) ^a	Source or reference
Strains		
<i>D. vulgaris</i> subsp. <i>vulgaris</i> Hildenborough	NCIMB 8303; isolated from clay soil near Hildenborough, United Kingdom	27
<i>D. vulgaris</i> SOD12	Plasmid p Δ SOD integrated into the chromosome; Suc ^s Cm ^r	This study
<i>D. vulgaris</i> SV2-6	Plasmid pSV2 integrated into the chromosome; Suc ^s Cm ^r	21
<i>D. vulgaris</i> SOD100	Δ sod; Suc ^r Cm ^r	This study
<i>D. vulgaris</i> RBR100	Δ rbr; Suc ^r Cm ^r	This study
<i>D. vulgaris</i> L2	Δ sor; Suc ^r Cm ^r	42
<i>E. coli</i> S17-1	<i>thi pro hsdR hsdM⁺ recA</i> RP4-2 (Tc::Mu Km::Tn7)	35
<i>E. coli</i> M15[pREP4]	Host strain for overexpression of pQE-encoded genes; Km ^r	Qiagen, Inc.
Plasmids		
pUC19Cm	pUC19 containing the <i>cat</i> gene	8
pNOT19	Cloning vector pUC19; <i>Nde</i> I site replaced by a <i>Not</i> I site	33
pMOB2	Contains <i>oriT</i> of plasmid RP4 and <i>Bacillus subtilis</i> <i>sacBR</i> genes on a 4.5-kb <i>Not</i> I fragment; Km ^r Cm ^r	33
pLITMUS28	Cloning vector; Ap ^r	New England Biolabs, Inc.
pLITSOD, p2SODCat, p2NotSODCat, and p Δ SOD		This study (see text)
pSV2		21
pQE30	Overexpression vector with an N-terminal His tag; Ap ^r	Qiagen, Inc.
pQS10		This study
Primers		
P154-f	5'-GAGGATACCGTGTATGC (5-21)*	
P155-r	5'-CAAAGGCCGGGAGTGTG (960-944)*	
P161-r	5'-CATGCCTGCCAGTAAAG (479-461)*	
P162-f	5'-CGGGAGCGGAGGGAC (484-499)*	
P175-f	5'-cctctagaCTCAACAAGGCCGTGGCGGG (336-355)*	
P176-r	5'-ccgcacGCGTGCTCCCATACGTGCGATGG (730-709)*	
P182-f	5'-GCGGGCTTGACAGTCTCCTCC (665-685)†	
P183-r	5'-GGGATGAGGTTGGAGGGATGC (1139-1119)†	
P184-f	5'-GCATCCCTCCAACCTCATCCC (1119-1139)†	
P185-r	5'-GAGTTCGAAGTGCCTTGGG (1714-1694)†	
P204-f	5'-cgggatCCATGCCGCCCCCTGCCCTATCCC (232-254)*	
P205-r	5'-ggggatcCTAGAGGCGTGTGCCACC (824-806)*	

^a *, Nucleotide positions in the 971-bp sequence containing the *sod* gene of *D. vulgaris* (accession number AF034841 [20]); †, nucleotide positions in the 3,270-bp sequence of the *rbr* operon of *D. vulgaris* (accession number M77011 [22]). Suc^r, sucrose resistance; Cm^r, CHL resistance; Ap^r, ampicillin resistance; Km^r, kanamycin resistance. Lowercase letters in primer sequences indicate added restriction endonuclease cleavage sites

Other immunoblotting reagents, including nitroblue tetrazolium and BCIP (5-bromo-4-chloro-3-indolylphosphate), as well as sequencing-grade modified trypsin, were from Promega. Reagent-grade chemicals were from either BDH, Fisher, or Sigma. Deoxyoligonucleotide primers were obtained from University Core DNA Services of the University of Calgary.

Bacterial strains, plasmids, and growth conditions. Strains, plasmids, and primers used or constructed in the present study are listed in Table 1. *Escherichia coli* and *D. vulgaris* strains were grown as described elsewhere (8, 26, 41).

Construction of *sod* and *rbr* deletion mutants. A 956-bp fragment containing the *sod* gene of *D. vulgaris* (Fig. 1) was amplified by PCR from genomic DNA by using the primers P154-f and P155-r and then cloned in the *Eco*RV site (GAT ↓ ATC) of pLITMUS28 to create plasmid pLITSOD. Plasmid pLITSOD was PCR amplified with primers P161-r and P162-f. This deleted bp 480 to 483 of the sequence with accession number AF034841. The PCR product was ligated with the 1.4-kb *Bam*HI fragment from pUC19Cm to yield plasmid p2SODCat. The 2.4-kb insert of this plasmid was transferred to pNOT19 after digestion of both with *Kpn*I and *Pst*I to give plasmid p2NotSODCat. The suicide vector p Δ SOD (9.6 kb) was generated by inserting the 4.5-kb *Not*I fragment from pMOB2 into the *Not*I site of plasmid p2NotSODCat. This plasmid was transferred to *D. vulgaris* by conjugation with *E. coli* S17-1(p Δ SOD). A single cross-over integrant, *D. vulgaris* SOD12, was selected. Growth of *D. vulgaris* SOD12 in the presence of chloramphenicol (CHL) and sucrose (8) yielded *D. vulgaris* SOD100 (Fig. 1). For generation of an *rbr* deletion mutant, suicide plasmid pSV2, containing the *cat* gene flanked by regions upstream and downstream from the *rbr* gene and constructed as described elsewhere (21), was transferred to *D. vulgaris* by conjugation, and a single crossover integrant *D. vulgaris* SV2-6 was

selected. Treatment of *D. vulgaris* SV2-6 with CHL and sucrose gave replacement mutant *D. vulgaris* RBR100 (Fig. 1).

Southern blot and PCR analysis for genotypic verification. Replacement mutagenesis of the *sod* and *rbr* genes was verified by PCR with a Perkin-Elmer Gene Amp 2400 PCR system with *Taq*I DNA polymerase and primer pairs indicated in Fig. 1 and Table 1. Replacement mutagenesis of the *rbr* gene was also verified by Southern blotting (43) by using a probe generated by PCR with primers P182-f and P183-r and labeled with [α -³²P]dCTP by the random hexamer procedure. Radioactive images of the blots were displayed with a Fuji BAS1000 Bioimaging Analyzer.

Immunoblotting. Expression of *sod* and *rbr* genes was demonstrated by immunoblotting. A rabbit polyclonal antibody against Rbr, purified from *D. vulgaris*, was obtained from Don Kurtz, Jr., Department of Chemistry, University of Georgia. His-tagged Fe-Sod from *D. vulgaris* was overproduced in *E. coli* M15[pREP4](pQS10) with the QIAexpress system (Qiagen). Plasmid pQS10 was constructed by PCR amplification of the *sod* gene from plasmid pLITSOD with primers p204-f and p205-r and inserting the PCR product into plasmid pQE30 (Qiagen) after cleavage with *Bam*HI and *Kpn*I. Immunoreactive sera of suitable titers were obtained by injecting two mice intraperitoneally on days 1 and 26 with 400 μ g of purified His-tagged Fe-Sod. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were carried out as described elsewhere (15, 26, 38).

Sod activity staining. Early-stationary-phase cultures in 100 ml of medium C (27) were harvested by centrifugation (20 min, 4,000 \times g) and washed once with 100 mM Tris-HCl (pH 8.0). The cell pellet was then resuspended in 0.8 ml of 100 mM Tris-HCl-100 mM EDTA (pH 9.0) (TE) and incubated at 37°C for 30 min

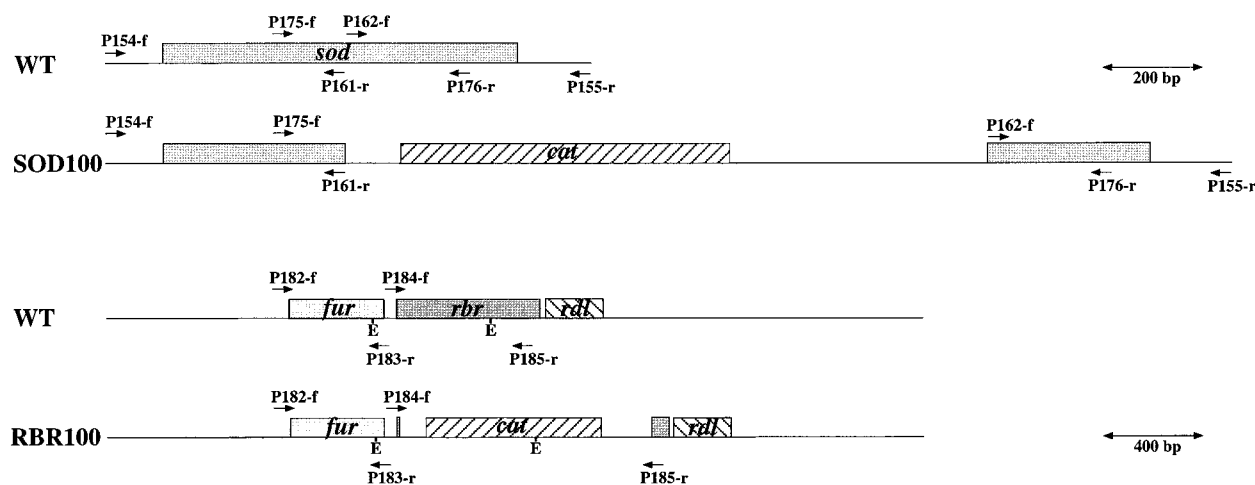


FIG. 1. Maps for the *rbr* and *sod* regions. A scale of 200 bp is indicated for maps for *D. vulgaris* wild type (WT) and the marker replacement mutant *D. vulgaris* SOD100, as well as the hybridization positions of primers P154-f, P155-r, P161-r, P162-f, P175-f, and P176-r and the locations of the *sod* and *cat* genes. A scale of 400 bp is indicated for *D. vulgaris* wild type and the marker replacement mutant *D. vulgaris* RBR100, as well as the hybridization positions of primers P182-f, P183-r, P184-f, and P185-r; the locations of *fur*, *rbr*, *rdl*, and *cat* genes; and the positions of restriction sites for *EcoRI* (E).

(25, 39). After centrifugation ($15,000 \times g$, 15 min at 6°C), the red supernatant (the TE-extract) was transferred into a clean microfuge tube. The TE pellet was resuspended in 3 ml of TE and lysed by two passes through a French press minicell at $1,800 \text{ lb/in}^2$. Proteins were quantified by using the DC protein assay (Bio-Rad). Appropriate amounts of proteins were frozen in liquid nitrogen, dried in a speed vacuum, and then dissolved in $10 \mu\text{l}$ of H_2O plus $10 \mu\text{l}$ of native protein sample buffer (100 mM Tris-Cl [pH 6.8], 20% glycerol, 0.2% bromophenol blue). Samples were loaded onto 12% (wt/vol) nondenaturing minigels and run at 4°C . Sod activity bands were located by staining as described elsewhere (2). Inhibition of the Sod activity in native polyacrylamide gels was tested by adding 10 mM azide (NaN_3), cyanide (KCN), or hydrogen peroxide (H_2O_2) to solutions used for Sod activity staining. Sod activities were quantified spectrophotometrically by using the xanthine oxidase-cytochrome *c* system described by McCord and Fridovich (23).

Exposure to air. Cultures (5 ml) of wild-type and mutant strains were grown anaerobically in medium C with kanamycin ($25 \mu\text{g/ml}$). At time zero, 0.5 ml of these cultures was diluted into 50 ml of aerobic medium C without yeast extract and then stirred with a magnetic stirrer continuously in air at room temperature. Also, $10 \mu\text{l}$ of anaerobic cultures were serially diluted in medium C. Aliquots ($100 \mu\text{l}$) of the 10^5 - and 10^6 -fold-diluted cultures were spread on medium E plates (27) to obtain the zero time count of CFU/ml. Aliquots (0.5 ml) of the air-exposed cultures were periodically transferred to the anaerobic hood, serially diluted, and plated. Colonies were counted after 1 week of anaerobic incubation at 32°C as the number of surviving CFU/ml. Data collected over a 72-h incubation period were fitted to the equation $\ln(N_t/N_0) = -kt$, where N_t and N_0 are the recorded CFU/milliliter at times zero and t , respectively, and k is the first-order inactivation rate constant (per hour). Only k values derived from experiments for which the data fitted a straight line with a correlation coefficient $r > 0.85$ were used. Alternatively, colonies present on medium E plates after 5 days of anaerobic incubation at 32°C were exposed to air for 30, 50, or 66 h. At each of these times the plates were briefly returned to the hood. Part of a colony was streaked on a new medium E plate, whereas the other part was inoculated into 5 ml of anaerobic liquid medium C. Growth on the plates and in the liquid medium was scored after 7 and 5 days, respectively.

Exposure to increased concentrations of internally generated superoxide. Experimental procedures were the same as described for exposure to air, except that 0.1 mM paraquat was included in the 50 ml of aerated medium into which 0.5 ml of the anaerobically grown cells was diluted.

Exposure to externally generated superoxide. Aliquots ($100 \mu\text{l}$) of anaerobic cultures in medium C with kanamycin were pipetted into 1.5-ml sterile microfuge tubes in the anaerobic hood. The tubes were transferred to air and, after 30 min, xanthine and xanthine oxidase were added to final concentrations of 0.22 mM and 0.45 U/ml, respectively. After they were left in air for an additional 30 to 60 min, the tubes were returned to the anaerobic hood and serially diluted, and $100\text{-}\mu\text{l}$ portions of appropriate dilutions were spread on medium E plates. The fraction (f) of surviving cells was calculated by dividing the surviving CFU/

milliliter by the count for unexposed aliquots. A time course for superoxide inactivation was done by transferring $30\text{-}\mu\text{l}$ aliquots of anaerobic cultures into 3 ml of aerobic medium C without lactate but containing xanthine (0.1 mM), xanthine oxidase (0.037 U/ml), and catalase (1 U/ml) and exposing the aliquots to air with shaking. Aliquots ($20 \mu\text{l}$) were removed after 5, 10, 15, and 30 min; transferred to the anaerobic chamber; and immediately plated onto medium E plates in duplicate. After incubation of the plates for 5 days at 32°C , colonies were counted, and the number of surviving CFU/milliliter was determined. This experiment was done in triplicate.

Exposure to H_2O_2 . Anaerobic cultures ($100 \mu\text{l}$, 10^8 cells/ml) were spread onto medium E plates with a glass spreader. Hydrogen peroxide ($20 \mu\text{l}$, 0.5 mM) was spotted onto a circular Whatman filter paper disk, which was placed on the cell lawn (20). The plates were incubated anaerobically for 4 days, and the diameter of the growth inhibition zone surrounding the disks was measured with a ruler.

Two-dimensional gel electrophoresis (2DE). Strains were grown in 50 ml of a defined lactate-sulfate medium (41, 44). TE-extracts prepared from log-phase cells were stored frozen at -80°C . For isoelectric focusing, 5 to $10 \mu\text{l}$ of TE-extract (20 to $40 \mu\text{g}$ of protein) diluted in 345 to $340 \mu\text{l}$ of urea-containing rehydration buffer (9) was focused with 18-cm Immobiline Drystrip gels (pH 3-10NL; Amersham Pharmacia Biotech). For the second dimension, proteins were separated on 12.5% (wt/vol) polyacrylamide gels containing 0.1% (wt/vol) SDS (29). After overnight electrophoresis at 20°C , the gels were fixed and then stained either with silver or Coomassie brilliant blue (CBB). Data acquisition of stained gels was performed by using the IMAGE Scanner (Amersham Pharmacia Biotech).

MALDI-TOF/MS. Selected protein spots were excised manually from either silver- or CBB-stained gels and placed in microfuge tubes. The gel pieces were washed, dried, and digested with trypsin solution (31, 45). Trypsin-digested samples were analyzed by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF/MS) on a Voyager DE-STR (Applied Biosystems, Foster City, Calif.). Peptide mass fingerprints were analyzed by using ProteinProspector software at <http://prospector.ucsf.edu> or were searched against a preliminary, annotated database of *D. vulgaris* provided by John Heidelberg from The Institute of Genomic Research by using the Knexus/Profound software package from Genomic Solutions.

RESULTS

Generation of marker replacement mutants. Gene replacement was achieved by treating cultures of the single crossover integrants, *D. vulgaris* SOD12 and *D. vulgaris* SV2-6, with sucrose in the presence of CHL. For *D. vulgaris* SOD12 the procedures outlined by Fu and Voordouw (8), with rich media, were followed. Only a single colony, representing replacement

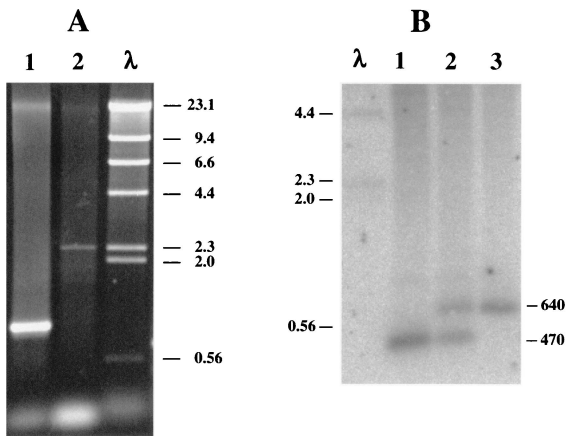


FIG. 2. (A) Agarose gel of PCR products of DNA from *D. vulgaris* wild-type (lane 1) and SOD100 (lane 2) strains with primers P154-f and P155-r. (B) Southern blot of *Eco*RI-digested chromosomal DNAs of *D. vulgaris* wild type (lane 1), strain SV2-6 (lane 2), and strain RBR100 (lane 3). The fragment sizes of a *Hind*III digest of bacteriophage λ DNA are indicated (lanes λ).

mutant *D. vulgaris* SOD100, was eventually obtained. For *D. vulgaris* SV2-6, use of these same procedures was unsuccessful, even after extensive screening. However, plating on defined hydrogen-sulfate plates containing sucrose and CHL (26) yielded five colonies, three of which had the desired genotype.

Verification of genotype of marker replacement mutants. Amplification of DNA from the SOD100 strain with primers P154-f and P155-r gave a 2.3-kb PCR product, whereas a 0.95-kb product was obtained with DNA from the wild-type strain (Fig. 2A), indicating insertion of the CHL resistance (Cm^r) marker. PCR of genomic DNA of the single crossover integrant yielded both products (not shown). Use of primers P175-f and P176-r (not shown) also confirmed the maps shown in Fig. 1. The genotype of the RBR100 strain was confirmed by Southern blotting (Fig. 2B). Hybridization of an *Eco*RI digest of wild-type DNA with the P182/P183 probe gave a 470-bp band (Fig. 2B, lane 1), whereas 470- and 640-bp bands were seen for DNA from the single crossover integrant, *D. vulgaris* SV2-6 (Fig. 2B, lane 2). The gene replacement mutant *D. vulgaris* RBR100 displayed only the 640-bp band (Fig. 2B, lane 3), in agreement with the maps shown in Fig. 1. Use of the P184-f and P185-r primer pair gave a 0.6-kb fragment for the wild-type, 0.6- and 1.1-kb fragments for the SV2-6, and a 1.1-kb fragment only for the RBR100 strain, respectively (data not shown). Immunoblotting confirmed the absence of the *sod* gene product in *D. vulgaris* SOD100 (data not shown) and of the *rbr* gene product in *D. vulgaris* RBR100 and their presence in the wild-type strain (Fig. 3).

Sod activity staining. Sod activity staining of nondenaturing gels showed a major band, near the top of the gel in the wild-type TE-extract (Fig. 4A, lane 2), which was absent in the SOD100 TE-extract (Fig. 4A, lane 1), indicating that this band represents Sod. A second, less-intense band was detected in the TE-extracts of both strains. It is not yet clear which protein this represents. Experiments with purified *D. vulgaris* Sor indicated it to run as a diffuse band lower in the gel (not shown). When TE-pellet samples were loaded, no activity bands were

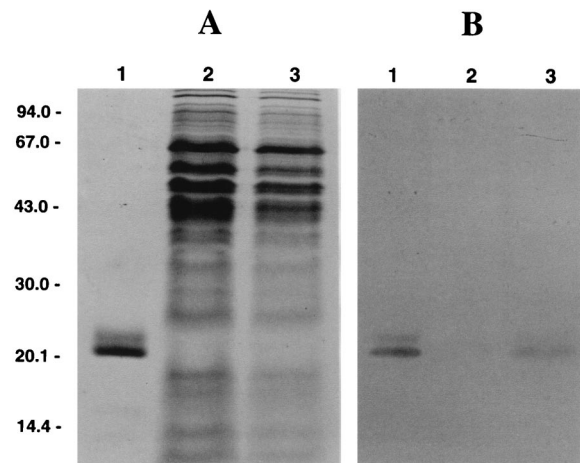


FIG. 3. Immunoblot analysis of Rbr content. (A) CBB-stained gel after SDS-PAGE. Lane 1, purified Rbr, 10 μg ; lane 2, RBR100 strain, 50 μg of protein; lane 3, wild-type strain, 30 μg of protein. (B) Immunoblot of gel as in panel A, except that only 1 μg of purified Rbr was loaded in lane 1.

detected for the *sod* mutant (Fig. 4A, lane 4), whereas a small amount of Sod (probably due to incomplete separation from TE-extract) was found for the wild type (Fig. 4A, lane 3). These results do not prove a periplasmic localization of Sod because TE-extracts also contain cytoplasmic proteins (Table 4). Thus, evidence for a periplasmic localization is primarily provided by the presence of an N-terminal, twin-arginine signal peptide (20). When more TE-extract protein was loaded, a weakly staining band was observed in the lower part of the gel for wild-type and *sor* and *sod* mutant strains but not for the RBR100 strain (data not shown). This could thus represent Rbr. Lehman et al. (17) also reported that Rbr can be identified on native gels by Sod activity staining. Rbr can remove superoxide under the conditions used for the staining (2), even though the protein is neither a Sod nor a Sor.

Sod activity, as monitored by staining of native polyacrylamide gels, was observed in the presence of cyanide (Fig. 4B,

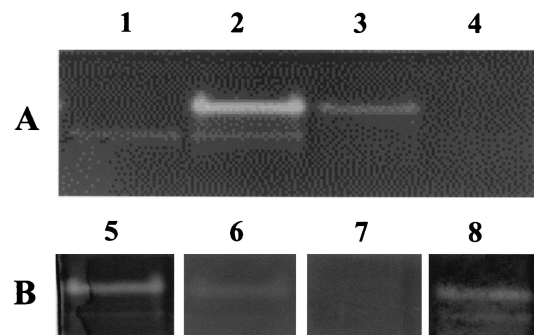


FIG. 4. Sod fractionation and sensitivity of Sod activity staining to inhibitors. Samples (75 μg of protein in all lanes 1 to 8) were run on a native 12% (wt/vol) polyacrylamide gel. (A) Fractionation into TE-extracts from SOD100 (lane 1) and wild-type (lane 2) strains and TE-pellets from wild-type (lane 3) and SOD100 (lane 4) strains. (B) Sensitivity to inhibitors as shown by activity staining without inhibitors (lane 5) or in the presence of 10 mM NaN_3 (lane 6), 10 mM H_2O_2 (lane 7), or 10 mM KCN (lane 8).

TABLE 2. Effect of various oxidative stresses on *D. vulgaris* wild-type and mutant strains

Stress ^a	Strain	Parameter ^b	n ^c	Mean \pm avg deviation ^d
Air	Wild type	<i>k</i>	10	0.083 \pm 0.025 h ⁻¹
	Sod100 (<i>sod</i>)	<i>k</i>	8	0.092 \pm 0.025 h ⁻¹
	L2 (<i>sor</i>)	<i>k</i>	4	0.272 \pm 0.050 h ⁻¹
	Rbr100 (<i>rbr</i>)	<i>k</i>	4	0.073 \pm 0.032 h ⁻¹
Air+PQ	Wild type	<i>k</i>	4	0.083 \pm 0.037 h ⁻¹
	Sod100 (<i>sod</i>)	<i>k</i>	3	0.124 \pm 0.046 h ⁻¹
	L2 (<i>sor</i>)	<i>k</i>	6	0.304 \pm 0.074 h ⁻¹
	Rbr100 (<i>rbr</i>)	<i>k</i>	4	0.110 \pm 0.060 h ⁻¹
Air+X+XO	Wild type	<i>f</i>	10	39% \pm 13%
	Sod100 (<i>sod</i>)	<i>f</i>	10	28% \pm 11%
	L2 (<i>sor</i>)	<i>f</i>	10	15% \pm 3%
	Rbr100 (<i>rbr</i>)	<i>f</i>	10	35% \pm 14%
H ₂ O ₂	Wild type	<i>d</i>	11	14.7 \pm 0.8 mm
	Sod100 (<i>sod</i>)	<i>d</i>	11	15.3 \pm 0.9 mm
	L2 (<i>sor</i>)	<i>d</i>	11	14.3 \pm 1.0 mm
	Rbr100 (<i>rbr</i>)	<i>d</i>	11	14.7 \pm 1.0 mm

^a Air is a 100-fold dilution into air-saturated medium; Air+PQ is a 100-fold dilution into air-saturated medium containing 0.1 mM paraquat; Air+X+XO is exposure to air and xanthine plus xanthine oxidase for 30 to 60 min; H₂O₂ is exposure to 10 μ M of H₂O₂ applied to a disk on a lawn of bacteria.

^b *k* is the first-order inactivation constant; *f* is the fraction of surviving bacteria determined for a 0.5- to 1-h exposure in Air+X+XO; *d* is the diameter of the zone without growth surrounding the disk on which H₂O₂ was placed.

^c *n* is the number of experiments.

^d Mean is the mean value for *n* experiments. Deviation is the average deviation from the mean for *n* experiments.

lane 8) but not in the presence of H₂O₂ (Fig. 4B, lane 7), whereas only a slight stain was observed in the presence of azide (Fig. 4B, lane 6), indicating significant inhibition. These data suggest that *D. vulgaris* Sod is an iron-containing enzyme, similar to that of *D. gigas* (6). Fe-Sod is sensitive to H₂O₂ and azide but not to cyanide, whereas Mn-Sod is only inhibited by azide and CuZn-Sod is sensitive to cyanide and H₂O₂.

Most of the Sod activity, measured as the amount of enzyme required to inhibit the reduction of cytochrome *c* by 50%, was found in the wild-type TE-extract (33.6 \pm 1.0 U/mg), whereas less activity was detected in the TE-pellet (9.1 \pm 0.6 U/mg). No activity was detected in the TE-extract of the *sod* mutant, whereas an activity similar to that observed for the wild type was detected in the TE-pellet (10.7 \pm 1.0 U/mg). Again, it is not clear which protein is represented by this activity, since the genome sequence does not indicate the presence of a second Sod.

Resistance to oxidative stress. Because no statistically valid differences were observed for anaerobically grown log-phase or early-stationary-phase cells, all data were averaged. Average rate constants *k* for inactivation of *D. vulgaris* after dilution into fully aerated medium were very similar for the wild-type, the *sod* mutant, and the *rbr* mutant strains. Only the *k* value for the *sor* mutant was significantly higher (Table 2, 0.083 and 0.272 h⁻¹, respectively). Surviving fractions can be calculated from these *k* values as follows: *f* = 14% for 24 h of air exposure of the wild-type, the *sod* mutant, and the *rbr* mutant strains and *f* = 0.15% for 24 h of air exposure of the *sor* mutant strain. Air exposure of colonies on plates had a more discriminating inactivating effect. The *sor* mutant was again the most sensitive

TABLE 3. Growth of single colonies of wild-type and mutant strains after exposure to air^a

Growth condition and time of exposure to air (h)	Growth of:			
	WT	Sod100 (<i>sod</i>)	L2 (<i>sor</i>)	Rbr100 (<i>rbr</i>)
Growth on plates				
30	++++	++++	---±	++++
50	+++±	+++±	----	+++±
66	+++±	----	----	+++±
Growth in liquid medium				
30	++++	+++±	+++±	++++
50	+++±	+++±	----	+++±
66	+++±	----	----	+++±

^a The results of four experiments are summarized. WT, wild type. Growth was scored as good (+), poor (±), or absent (–), after 7 days for plates and after 5 days for liquid medium.

and failed to grow even after 30 h of air exposure. However, both the *sor* and the *sod* mutants failed to grow after 66 h of air exposure, whereas growth of the wild-type and *rbr* mutant strains was observed in three of four and in two of four experiments, respectively (Table 3).

In aerobic bacteria the oxidative stress level can also be increased by addition of paraquat (methylviologen), which increases the rate of cytoplasmic superoxide production in *E. coli* (10). As indicated in Table 2, the addition of 0.1 mM paraquat left the inactivation rate constant *k* of the wild type unchanged, whereas it increased somewhat for the mutant strains, corresponding to 24-h survivals of 14, 5, 0.07, and 7% for the wild-type and *sod*, *sor*, and *rbr* mutant strains, respectively. The insensitivity of wild-type *D. vulgaris* to paraquat agrees with an earlier study (20), in which a larger effect of paraquat on the inactivation rate of the *sor* mutant was found. The increased sensitivity of the SOD100 strain was unexpected, because paraquat is generally thought to only increase the production of cytoplasmic superoxide, which does not serve as a substrate for a periplasmic Sod since superoxide is membrane impermeable.

Exposure of *D. vulgaris* cells to limited amounts of external superoxide (0.10 mM xanthine, 0.037 U of xanthine oxidase/ml) did not result in a loss of viability of the wild type, whereas both *sod* and *sor* mutants were affected even after short exposure times (Fig. 5). After 30 min, only 50% of the *sod* mutant cells survived. However, the surviving fraction of *sor* mutant cells was even lower (33%). When *D. vulgaris* cells were exposed to high concentrations of externally produced superoxide (0.22 mM xanthine, 0.45 U of xanthine oxidase/ml), all strains lost viability. Relative to the wild-type strain (Table 2, 39% survival), the *sor* mutant was again affected the most (15% survival). The *sod* mutant was affected less strongly (28% survival), whereas the *rbr* mutation had little effect (35% survival). Thus, periplasmic Fe-Sod provided protection to externally produced superoxide. The inactivation of the *sor* mutant is in part caused by air exposure, i.e., for *k* = 0.272 h⁻¹ a surviving fraction of *f* = 76% can be calculated for 1 h of exposure to air only. However, even after we corrected for that, cytoplasmically located Sor appears to be important for combating external superoxide stress.

Exposure to H₂O₂ did not present a lethal form of oxidative

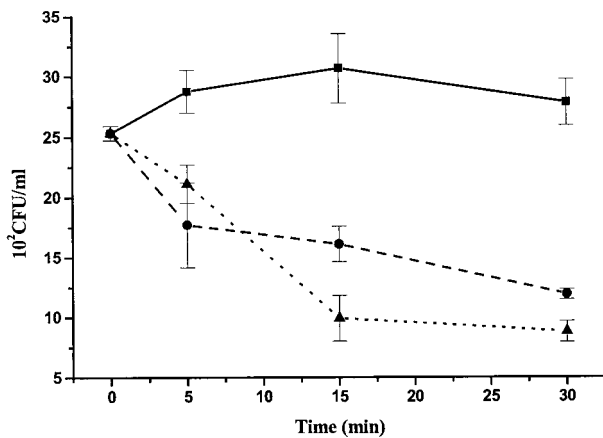


FIG. 5. Time course of loss of viability of the wild-type, SOD100, and L2 strains after exposure to limited amounts of extracellularly generated superoxide. Symbols: ■, wild type; ●, SOD100; ▲, L2.

stress under the chosen assay conditions (Table 2). The presence of several *rbr* homologs in the *D. vulgaris* genome (<http://www.tigr.org>) may contribute to the lack of H₂O₂ sensitivity.

2DE. Comparison of silver-stained gels for the wild-type and SOD100 strains indicated that spot 40 in the wild-type TE-extract (Fig. 6A) was absent from the TE-extract of the SOD100 strain (not shown). MALDI-TOF/MS analysis of the protein digest, combined with a search of the NCBI database with MS-Fit software, indicated spot 40 to be *D. vulgaris* Fe-Sod (Table 4). A 14-kDa protein in the wild-type TE-extract

(Fig. 6A, spot 39) was missing from the *sor* mutant proteome (Fig. 6B). Analysis of the trypsin-digested samples by MALDI-TOF/MS and comparison of the determined peptide masses with a database derived from the *D. vulgaris* genome by using the program ProFound (Genomic Solutions) indicated ORF04271 Sor as the top score. Several other proteins were cut out from the CBB-stained gel and successfully identified by MALDI (Table 4). The position of Rbr (21 kDa) could not be identified by comparing protein patterns in silver-stained 2DE gels obtained for the *rbr* mutant and wild-type strains. In view of the central role of Sor in resistance to oxygen stress of *D. vulgaris* under fully aerated conditions (Table 2), we determined the dependence of the 2DE pattern on the length of air exposure. Prolonged exposure to air (1 to 4 h) led to a decrease in intensity of Sor (spot 39) relative to neighboring proteins in the 2DE patterns (Fig. 7).

DISCUSSION

Reduction of oxygen entering the cytoplasm of *D. vulgaris* Hildenborough by soluble Roo or membrane-bound Cox or Cbd results in generation of the ROS superoxide and H₂O₂. Sor and Rbr have been proposed to remove these cytoplasmically generated ROS by reduction (20). Because superoxide is not membrane permeable, periplasmic SOD has been proposed to protect *D. vulgaris* from superoxide generated in the periplasm, when oxygen reacts with reduced periplasmic redox proteins such as hydrogenase or cytochrome *c*₃ (20). Interestingly, the *D. vulgaris* *roo* gene is located immediately down-

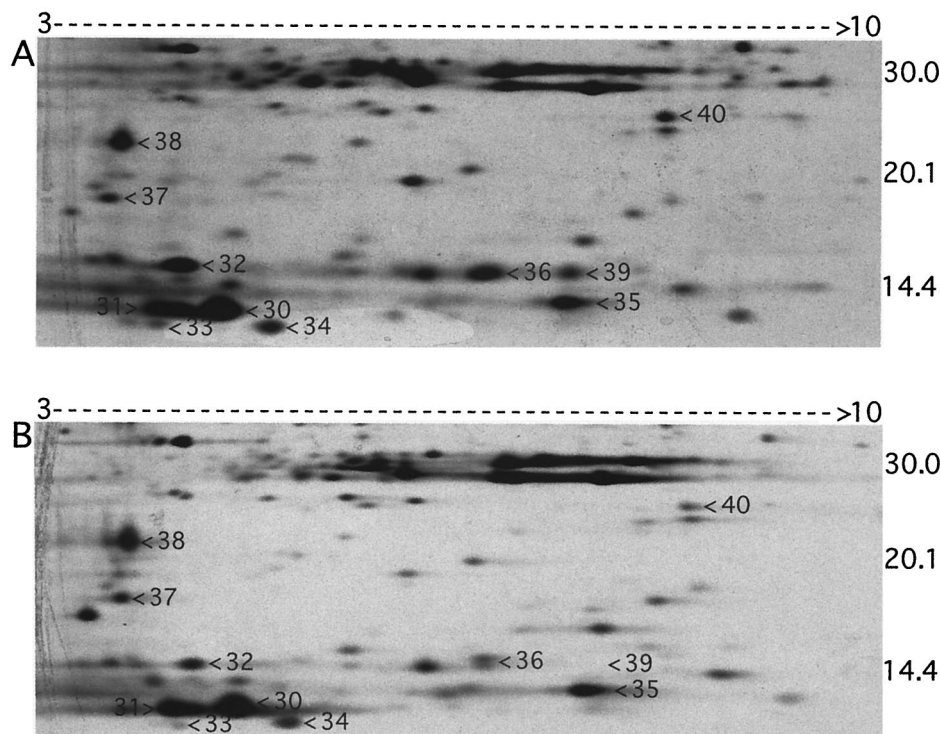


FIG. 6. 2DE gels of TE-extracts of *D. vulgaris* Hildenborough wild-type (A) and *sor* mutant (B) strains. Only a part of the silver-stained gels is shown. Proteins were separated on 18-cm IPG strips (pH 3-10NL; from left to right) and by 2DE according to the molecular mass (indicated in kilodaltons). The numbered protein spots were cut out from CBB-stained gels. Their MALDI identifications are presented in Table 4.

TABLE 4. MS identification of proteins from *D. vulgaris* separated by 2DE

<i>n</i> ^a	ID ^b	Sequence coverage ^c (%)	Significance ^d	pI ^e	<i>M</i> _r ^f	Location ^g	Name, possible function ^h
30	ORF03941	9	3.1 × 10 ⁻⁴	5.3	29.8	C	Hypothetical protein
31	ORF03581	28	0.054	5.1	12.0	C	Sulfite reductase, dissimilatory-type gamma subunit
32	ORFA00027	9	8.4 × 10 ⁻⁶	5.8	32.0	M	SrpA, putative
33	ORF02650	38	2.2 × 10 ⁻⁴	5.4	15.0	C	RNA-binding protein
34	ORF01017	49	6.4 × 10 ⁻⁵	5.4	10.2	C	RNA-binding protein
35	ORF02955	17	3.9 × 10 ⁻⁴	4.2	16.1	C	Hypothetical protein
36	ORF02453	28	3.5 × 10 ⁻¹³	5.7	12.6	C	Uncharacterized ACR, COG1433 family
37	ORF03883	52	5.9 × 10 ⁻⁹	4.9	17.0	C	Nitrogen assimilation regulatory protein
38	ORF00336	35	2.2 × 10 ⁻³	4.9	19.1	C	Adenylyl sulfate reductase, beta subunit
39	ORF04271	50	0.046	6.0	14.3	C	Sor, superoxide reductase
40	AF034841	36	1.437e ^{0.04}	6.6	22.1	P	Sod, superoxide dismutase

^a *n*, spot number in 2DE gels shown in Fig. 6.
^b ID, identification by MS in a database of coding genes for the *D. vulgaris* genome (<http://www.tigr.org>) provided by J. Heidelberg.
^c That is, the extent to which the combined peptide masses cover the indicated sequence.
^d That is, the significance of the match; probability that the match between the identified peptides and the indicated sequence is due to random chance.
^e pI is the average theoretical value calculated with programs found at <http://www.embl-heidelberg.de/cgi/pi-wrapper.pl> and at <http://www.biologie.uni-freiburg>.
^f *M*_r is the theoretical molecular mass derived from the genome sequence.
^g Most likely location—cytoplasmic (C), periplasmic (P), or in the membrane (M)—based on evaluation of the amino acid sequence.
^h Annotated function.
ⁱ Search was performed by using MS-Fit software (<http://prospector.ucsf.edu>) against the NCBI nr.6.6.2002 database. The indicated score is the MOWSE score.

stream from the *sor-rub* operon (<http://www.tigr.org>). Thus, Roo and Sor may collaborate in reduction and detoxification of oxygen entering the cytoplasm through use of rubredoxin (Rub) as a common intermediary electron donor.

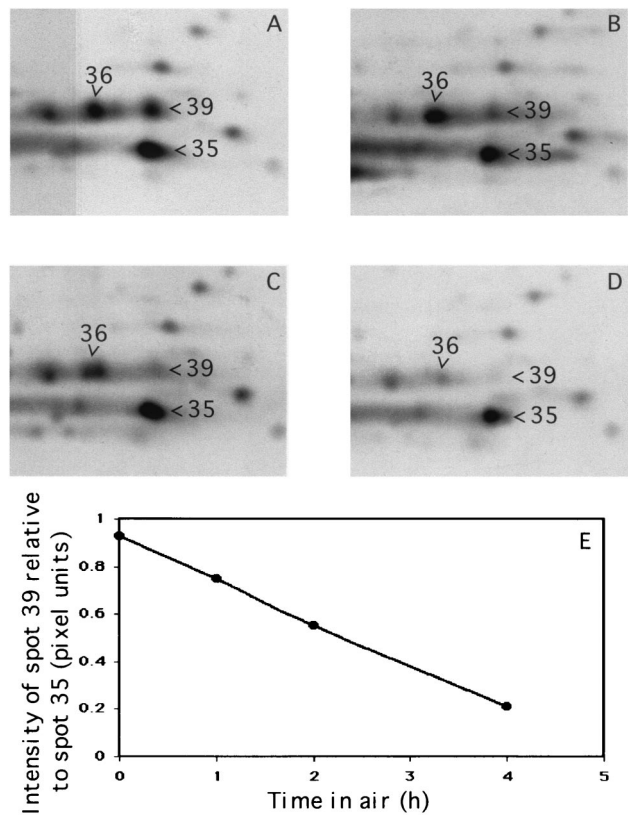


FIG. 7. Effect of air exposure on the *D. vulgaris* proteome. *D. vulgaris* wild type grown anaerobically in defined lactate-sulfate medium was exposed to air for 0 (A), 1 (B), 2 (C), and 4 (D) h. Part of the silver-stained 2DE gels of TE-extracts is shown. (E) Intensity of spot 39 (Sor) relative to that of spot 35 as a function of time.

The lack of a strong oxygen stress phenotype for the *rbr* mutant (Table 2) is not surprising in view of the presence of genes for two Rbr homologs, Rbr2 and nigerythrin (Ngr), in the genome (<http://www.tigr.org>). *D. vulgaris* also has a *kat* gene encoding catalase, but this is located on a *nif* gene-containing plasmid that is lost when cells are cultured in ammonium chloride-containing media as were used here (G. Voordouw, unpublished data). Thus, the wild-type level of H₂O₂ resistance of the *rbr* mutant (Table 2) was caused by Rbr2 and Ngr and not catalase.

A somewhat unexpected finding was that resistance to internally and externally generated superoxide was not cleanly separated in the *sor* and *sod* mutants. In the case of internally generated superoxide, it has been shown that the stable paraquat monocation radical (PQ^{•+}) formed from oxidized paraquat (PQ²⁺) in the cytoplasm can diffuse across the membrane, where it reacts with available oxygen to form superoxide (10). Hence, a role of Sod in remediating the additional oxidative stress induced by paraquat can be understood. The mechanism by which periplasmic superoxide induces additional oxidative stress in the cytoplasm is currently unknown but may be similar, i.e., through generation of membrane-transportable, lipophilic radicals.

Comparison of the sensitivity of *sor*, *sod*, and *rbr* mutants to oxidative stress indicates that under fully aerated conditions Sor is the key oxygen defense factor (Table 2). Apparently, superoxide is generated primarily in the cytoplasm under these conditions. The periplasmic redox protein pool may quickly drain electrons during exposure to fully aerated conditions as were used here, leaving no targets for periplasmic superoxide production. However, when superoxide is artificially generated outside the *D. vulgaris* cell, the presence of Sod appears to be advantageous, since the Sod mutant was found to be considerably more sensitive to such external superoxide stress than was the wild type (Table 2 and Fig. 5). Because *D. vulgaris* is not a pathogen, it is not exposed to large amounts of superoxide produced externally by other cells (e.g., macrophages). Sod is therefore more likely to function in the removal of periplas-

mic superoxide produced under microaerophilic conditions, when the periplasmic redox protein pool is at least partially reduced. Abdollahi and Wimpenny (1) have shown, in agreement with this conclusion, that in continuous cultures of *D. desulfuricans* Sod activity is maximal at 1% (vol/vol) oxygen. The activity was lower both in anaerobic cultures and in fully aerated cultures.

Periplasmic Sods in other microorganisms are either of the CuZn type (12, 32, 37) or the Mn type (16, 19). Although periplasmic CuZn-Sods have typical Sec system-dependent signal sequences, a twin-arginine leader sequence has been found in periplasmic Mn-Sod (19). To date, periplasmic Fe-Sods have only been found in *Desulfovibrio* spp. *D. vulgaris* Fe-Sod is exported through the Tat system (20), which only handles redox proteins with bound cofactors (40). The difference indicates that, prior to export, *Desulfovibrio* spp. assemble Fe-Sod in the cytoplasm, whereas metal acquisition by the enzymes exported via the Sec system is a periplasmic process. Evolution of cytoplasmically assembled, periplasmic Fe-Sod in *Desulfovibrio* spp. may have been necessitated by the fact that high sulfide concentrations produced by these organisms keep external concentrations of copper and zinc ions at extremely low levels. Fe-Sod or Mn-Sod in aerobic or facultative microorganisms, such as SodA and SodB in *E. coli*, are cytoplasmic enzymes. Because of their critical role in combating respiration-mediated superoxide stress, these are generally constitutive, whereas periplasmic CuZn-Sod is induced in stationary phase (12, 32, 37). A decreasing oxygen tension in stationary phase may induce oxygen-sensitive, periplasmic redox proteins (hydrogenases, nitrate reductases) associated with alternative respiratory chains, leading to increased periplasmic superoxide production. Hence, as in *D. vulgaris*, the need for periplasmic Sod in these other microorganisms may be greatest under conditions of low oxygen tension, when the periplasm has oxygen-sensitive targets.

In conclusion, it appears that under fully aerobic conditions Sor is the single most important oxygen defense factor. The decrease in Sor concentration recorded in wild-type cells during prolonged aeration (Fig. 7) may be caused by its inactivation upon continuous processing of highly reactive superoxide under conditions in which protein synthesis is compromised. This decrease may be a critical factor in causing the eventual cell death of *D. vulgaris* upon continued air exposure.

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M.F. and Y.Z. contributed equally to this study.

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