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 without 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 Escherichia 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 thermotrotophicum (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 A DNA were obtained from Pharmacia. [α-32P]ATP (10 mCi/μl; 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

<table>
<thead>
<tr>
<th>Strains, plasmid, or primer</th>
<th>Genotype, sequence (position), and/or comment(s)*</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. vulgaris</em> subsp. vulgaris Hildenborough</td>
<td>NCIMB 8303; isolated from clay soil near Hildenborough, United Kingdom</td>
<td>27</td>
</tr>
<tr>
<td><em>D. vulgaris</em> SOD12</td>
<td>Plasmid p2ΔSOD integrated into the chromosome; Suc* Cm'</td>
<td>This study</td>
</tr>
<tr>
<td><em>D. vulgaris</em> SV2-6</td>
<td>Plasmid pSV2 integrated into the chromosome; Suc* Cm'</td>
<td>21</td>
</tr>
<tr>
<td><em>D. vulgaris</em> SOD100</td>
<td>Δsod; Suc* Cm'</td>
<td>This study</td>
</tr>
<tr>
<td><em>D. vulgaris</em> RBR100</td>
<td>Δsod; Suc* Cm'</td>
<td>This study</td>
</tr>
<tr>
<td>E. coli S17-1</td>
<td>thi pro hsdR hsdM+ recA RP4-2 (Tc; Mu Km; Th7)</td>
<td>35</td>
</tr>
<tr>
<td>E. coli M15[pREP4]</td>
<td>Host strain for overexpression of pQE-encoded genes; Km'</td>
<td>Qiagen, Inc.</td>
</tr>
</tbody>
</table>

Plasmids

- pUC19Cm: pUC19 containing the cat gene
- pNOT19: Cloning vector pUC19; NotI site replaced by a NotI site
- pMOB2: Contains oriT of plasmid RP4 and Bacillus subtilis sacB genes on a 4.5-kb NotI fragment; Km' Cm'
- pLITMUS28: Cloning vector; Ap'
- pLITSOD, p2SODCat, p2NotSODCat, and p2ΔSOD: Construction of deletion mutants.
- pSV2: Overexpression vector with an N-terminal His tag; Ap'
- pQE30: This study
- p2NotSODCat, and p2ΔSOD: This study (see text)

Primers

- P154-f, P155-r, P161-r, P162-f, P182-f, P183-r, P184-f, P185-r: 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).
- P182-f, P183-r, and P184-f: Additional primers used to generate the Sod activity staining.

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

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 EcoRV site (GAT I) 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 BamHI fragment from pUC19Cm to yield plasmid p2SODCat. The 2.4-kb insert of this plasmid was transferred to pNOT19 after digestion of both with KpnI and PstI to give plasmid p2NotSODCat. The suicide vector p2ΔSOD (9.6 kb) was generated by inserting the 4.5-kb NotI fragment from pMOB2 into the NotI site of plasmid p2NotSODCat. This plasmid was transfected to *D. vulgaris* by conjugation with *E. coli* colI S17-1(p2ΔSOD). A single crossover integrant, *D. vulgaris* SOD12, was selected. Growth of *D. vulgaris* SOD12 in the presence of chloramphenicol (CHL) and sucrose (Suc) yielded *D. vulgaris* SOD100 (Fig. 1). For generation of an rbr deletion mutant, suicide plasmid p2SODCat was obtained from Don Kurtz, Jr., Department of Chemistry, University of Georgia. His-tagged Fe-Sod from *D. vulgaris* was obtained 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 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 BamHI and KpnI. Immunoreactive sera of suitable titers were obtained by injecting two mice intraperitoneally on days 1 and 26 with 400 μg 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 × 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 fur 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).

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 isolectric focusing, 5 to 10 µl of TE-extract (20 to 40 µg of protein) diluted in 345 to 340 µl 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 Coomasie 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 Kneusx/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 milliliter by the count for unexposed aliquots. A time course for superoxide inactivation was done by transferring 30-µl aliquots of anaerobic cultures into 3 ml of aerobic medium C without lactate but containing xanthine (0.1 mM), xanthine oxidase (0.037 U/µl), and catalase (1 U/ml) and exposing the aliquots to air with shaking. Aliquots (20 µl) 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 H2O2. Anaerobic cultures (100 µl, 10^8 cells/ml) were spread onto medium E plates with a glass spreader. Hydrogen peroxide (20 µl, 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 µl of TE-extract (20 to 40 µg of protein) diluted in 345 to 340 µl 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 Coomasie brilliant blue (CBB). Data acquisition of stained gels was performed by using the IMAGE Scanner (Amersham Pharmacia Biotech).
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 (Cmr) 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 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 polycrylamide gels, was observed in the presence of cyanide (Fig. 4B, 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).

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

<table>
<thead>
<tr>
<th>Stress</th>
<th>Strain</th>
<th>Parameter</th>
<th>n</th>
<th>Mean ± avg deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>Wild type</td>
<td>k</td>
<td>10</td>
<td>0.083 ± 0.025 h⁻¹</td>
</tr>
<tr>
<td></td>
<td>Sod100 (sod)</td>
<td>k</td>
<td>8</td>
<td>0.092 ± 0.025 h⁻¹</td>
</tr>
<tr>
<td></td>
<td>L2 (sor)</td>
<td>k</td>
<td>4</td>
<td>0.272 ± 0.050 h⁻¹</td>
</tr>
<tr>
<td></td>
<td>Rbr100 (rbr)</td>
<td>k</td>
<td>4</td>
<td>0.073 ± 0.032 h⁻¹</td>
</tr>
<tr>
<td>Air + PQ</td>
<td>Wild type</td>
<td>k</td>
<td>4</td>
<td>0.083 ± 0.037 h⁻¹</td>
</tr>
<tr>
<td></td>
<td>Sod100 (sod)</td>
<td>k</td>
<td>3</td>
<td>0.124 ± 0.046 h⁻¹</td>
</tr>
<tr>
<td></td>
<td>L2 (sor)</td>
<td>k</td>
<td>6</td>
<td>0.304 ± 0.074 h⁻¹</td>
</tr>
<tr>
<td></td>
<td>Rbr100 (rbr)</td>
<td>k</td>
<td>4</td>
<td>0.110 ± 0.060 h⁻¹</td>
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<tr>
<td>H₂O₂</td>
<td>Wild type</td>
<td>d</td>
<td>11</td>
<td>14.7 ± 0.8 mm</td>
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<td></td>
<td>Sod100 (sod)</td>
<td>d</td>
<td>11</td>
<td>15.3 ± 0.9 mm</td>
</tr>
<tr>
<td></td>
<td>L2 (sor)</td>
<td>d</td>
<td>11</td>
<td>14.3 ± 1.0 mm</td>
</tr>
<tr>
<td></td>
<td>Rbr100 (rbr)</td>
<td>d</td>
<td>11</td>
<td>14.7 ± 1.0 mm</td>
</tr>
</tbody>
</table>

- *A* 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 μmol of H₂O₂ applied to a disk on a lawn of bacteria.
- *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.
- *n* is the number of experiments.
- *Mean* is the mean value for *n* experiments. Deviation is the average deviation from the mean for *n* experiments.

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 (methyliodigen), 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. 3). 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 extran nal superoxide stress.

Exposure to *H₂O₂* did not present a lethal form of oxidative

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

<table>
<thead>
<tr>
<th>Growth condition and time of exposure to air (h)</th>
<th>Growth of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
</tr>
<tr>
<td>Growth on plates</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>66</td>
</tr>
<tr>
<td>Growth in liquid medium</td>
<td>30</td>
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<tr>
<td></td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>66</td>
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* 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.
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 H$_2$O$_2$ 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 NCBInr 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 H$_2$O$_2$. 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-

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**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.

**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.
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.

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. Voor-douw, 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 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 (PO2−) formed from oxidized paraquat (PO2+−) 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-

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

<table>
<thead>
<tr>
<th>n</th>
<th>ORF</th>
<th>Sequence coverage (%)</th>
<th>Significance</th>
<th>pI</th>
<th>Mw</th>
<th>Location</th>
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<td>30</td>
<td>ORF03941</td>
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<td>22.1</td>
<td>C</td>
<td>Sod, superoxide dismutase</td>
</tr>
</tbody>
</table>

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 Mw is the theoretical molecular mass derived from the genome sequence. f Most likely location—cytoplasmic (C), periplasmic (P), or in the membrane (M)—based on evaluation of the amino acid sequence. g Annotated function. h Location 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.de. 

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.
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 cytoplastically 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 Sod is the single most important oxygen defense factor. The decrease in Sod 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.

ACKNOWLEDGMENTS

This work was supported by a grant from the Natural Science and Engineering Research Council of Canada (NSERC) to G.V. A database of the D. vulgaris Hildenborough genome was searched at the web site of The Institute for Genomic Research at http://www.tigr.org. Sequencing of this genome is financially supported by the U.S. Department of Energy. M.F. and Y.Z. contributed equally to this study. We thank Don Kurtz, Jr., and Heather Lumppio, Department of Chemistry, University of Georgia, for generating D. vulgaris SV2-6 and for polyclonal antibodies recognizing Rbr and D. Moinier from the IBSN Proteomics Facility for performing the SOD MALDI-TOF analysis. We thank John Heidelberg for making a preliminary annotation of the D. vulgaris genome available to us.

REFERENCES


