JOURNAL OF BACTERIOLOGY, Aug. 2007, p. 5996–6010 Vol. 189, No. 16
0021-9193/07/$08.00+0 doi:10.1128/JB.00368-07

Cell-Wide Responses to Low-Oxygen Exposure in *Desulfovibrio vulgaris* Hildenborough[▽]†

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Received 12 March 2007/Accepted 20 May 2007

The responses of the anaerobic, sulfate-reducing organism *Desulfovibrio vulgaris* Hildenborough to low-oxygen exposure (0.1% O_2) were monitored via transcriptomics and proteomics. Exposure to 0.1% O_2 caused a decrease in the growth rate without affecting viability. Concerted upregulation of the predicted peroxide stress response regulon (PerR) genes was observed in response to the 0.1% O_2 exposure. Several of the candidates also showed increases in protein abundance. Among the remaining small number of transcript changes was the upregulation of the predicted transmembrane tetraheme cytochrome c_3 complex. Other known oxidative stress response candidates remained unchanged during the low-O_2 exposure. To fully understand the results of the 0.1% O_2 exposure, transcriptomics and proteomics data were collected for exposure to air using a similar experimental protocol. In contrast to the 0.1% O_2 exposure, air exposure was detrimental to both the growth rate and viability and caused dramatic changes at both the transcriptome and proteome levels. Interestingly, the transcripts of the predicted PerR regulon genes were downregulated during air exposure. Our results highlight the differences in the cell-wide responses to low and high O_2 levels in *D. vulgaris* and suggest that while exposure to air is highly detrimental to *D. vulgaris*, this bacterium can successfully cope with periodic exposure to low O_2 levels in its environment.

Sulfate-reducing bacteria (SRB) like *Desulfovibrio* spp. are truly cosmopolitan organisms that flourish in deep subsurface sediments, rice paddies, lake and ocean sediments, insect and animal guts, sewers, and oil pipelines (8, 27, 40, 41, 51). Although considered obligate anaerobes for many years after their discovery, *Desulfovibrio* spp. are found in many environments that are regularly or periodically exposed to oxygen (8, 20, 35). A number of *Desulfovibrio* spp. have been documented to reduce millimolar levels of O_2 (12), and in an O_2 gradient *Desulfovibrio vulgaris* Hildenborough localizes to very low O_2 concentrations rather than the anoxic region (30). However, *D. vulgaris* does not couple growth to O_2 respiration (8, 12), and even small amounts of O_2 affect growth adversely (57). Although *D. vulgaris* has been shown to survive long periods of air exposure (8, 9), it grows optimally in an anaerobic environment (46).

Several studies have focused on discovering the *D. vulgaris* genes involved in its oxidative stress response (7, 36), and a basic model for O_2 stress response in *D. vulgaris* has been proposed and reviewed (7, 37). *D. vulgaris* has two major mechanisms for superoxide removal, namely, the superoxide reductase (Sor) and the superoxide dismutase (Sod). The gene encoding Sor, also called desulfoferrodoxin or rubredoxin oxidoreductase (*rbo*), occurs as part of an operon that also encodes a rubredoxin (*rub*) and the rubredoxin oxygen oxidoreductase (*roo*). The Sor reportedly works in conjunction with peroxidases (e.g., AhpC and rubrerythrins [18, 37]) and electron transfer proteins such as rubredoxins (7) to convert superoxides to water. For reactive oxygen species (ROS) removal, the Sor mechanism is considered to be the preferred pathway as it does not regenerate any intracellular O_2 (14, 26, 28, 42). The *D. vulgaris* genome includes multiple genes, such as genes encoding rubrerythrins, rubredoxins, and a nigerytherin, that are anticipated to be involved in peroxide reduction (Fig. 1). Sequence analysis of the *D. vulgaris* genome (23) enabled prediction of regulons, among which a putative PerR regulon was defined (49). The inferred PerR regulon contains the *perR* regulator and a subset of the peroxide reduction genes mentioned above (*ahpC*, *rbr*, *rbr2*, *rdl*, and a gene encoding

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† Supplemental material for this article may be found at http://jb.asm.org/.

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▽ Published ahead of print on 1 June 2007.

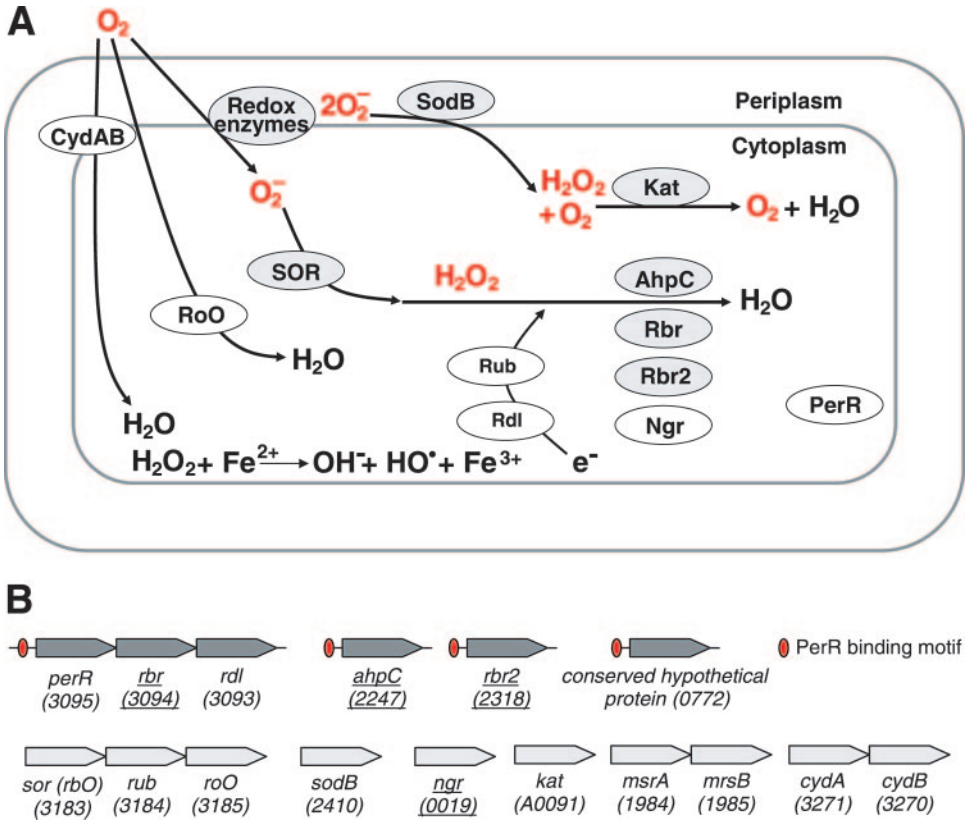

FIG. 1. Overview of selected O_2-responsive proteins in *D. vulgaris*. (A) Localization and mechanistic roles of individual proteins in O_2 reduction in the gram-negative *D. vulgaris* cell. While all candidates are represented in the transcriptome data, those for which proteomics data were available are shaded. Also shown is the Fenton's reaction between Fe^{2+} and H_2O_2, which generates harmful hydroxyl radicals. (B) Predicted PerR regulon (candidates with potential PerR binding motifs) and other selected candidates. The underlined genes are reported to encode NADH peroxidases. DVU numbers are shown in parentheses.

conserved hypothetical protein [Fig. 1]). The *D. vulgaris* genome also encodes an Fe-Sod that has been shown to provide a protective mechanism in the periplasmic space where O_2-sensitive enzymes, such as the Fe hydrogenase (HydA/B), function (17, 54). The *D. vulgaris* Sod may also work in conjunction with a catalase, an efficient enzyme that catalyzes the turnover of H_2O_2 to water and oxygen (42). Interestingly, the *D. vulgaris* catalase is encoded on a 202-kb plasmid, which has been documented to be lost during growth in ammonium-rich medium (18).

Despite these protective mechanisms, ROS, such as superoxides and peroxides, are still produced during O_2 reduction and trigger a variety of cellular damage in both aerobic and anaerobic organisms (37, 45, 53). While it is the ROS that cause the majority of O_2-related damage, O_2 itself also irreversibly deactivates critical periplasmic proteins, such as reduced Fe hydrogenases (54). Oxidative stress due to O_2 exposure is known to have multiple effects on cellular physiology, and exposure to O_2 at both high and low levels can be expected to elicit cellular responses, especially for anaerobic organisms. Our current knowledge of the oxidative stress response mechanisms in *D. vulgaris* is derived mainly from studies conducted using exposure to air or 100% O_2 (13, 16–18, 59). A survey of these studies also revealed that differences in experimental protocols led to important differences in cellular responses. For example, a study of oxygen-responsive genes in *D. vulgaris* (18) reported a loss of viability in response to air exposure, yet a similar microarray study of air exposure (59) observed no such loss. Further, the modulation of the multiple protective mechanisms in response to low-O_2 exposure was not explored. The specificity of many of these mechanisms in O_2 exposure also remains undefined, as many of the candidate proteins are intimately linked with the redox status of the cell and may have redundant functions.

We hypothesized that a cell-wide study of *D. vulgaris* in a low-oxygen environment might uncover new information about these mechanisms. Consistent with this, a recent study showed a *roo* mutant to be sensitive to 0.2% O_2 exposure (57). Cell-wide data from an air stress response study may provide the perspective required to determine the specificity of responses to low-O_2 exposure. In order to minimize the variability resulting from experimental setup and to place our data in the context of previous studies, we conducted controlled experiments to measure *D. vulgaris* responses to both low oxygen levels and air.

MATERIALS AND METHODS

Bacterial growth and maintenance. Bacterial strains were grown and maintained as described previously (39). In brief, *D. vulgaris* Hildenborough (ATCC 29579) was grown in a defined lactate (60 mM)-sulfate (50 mM) medium, LS4D

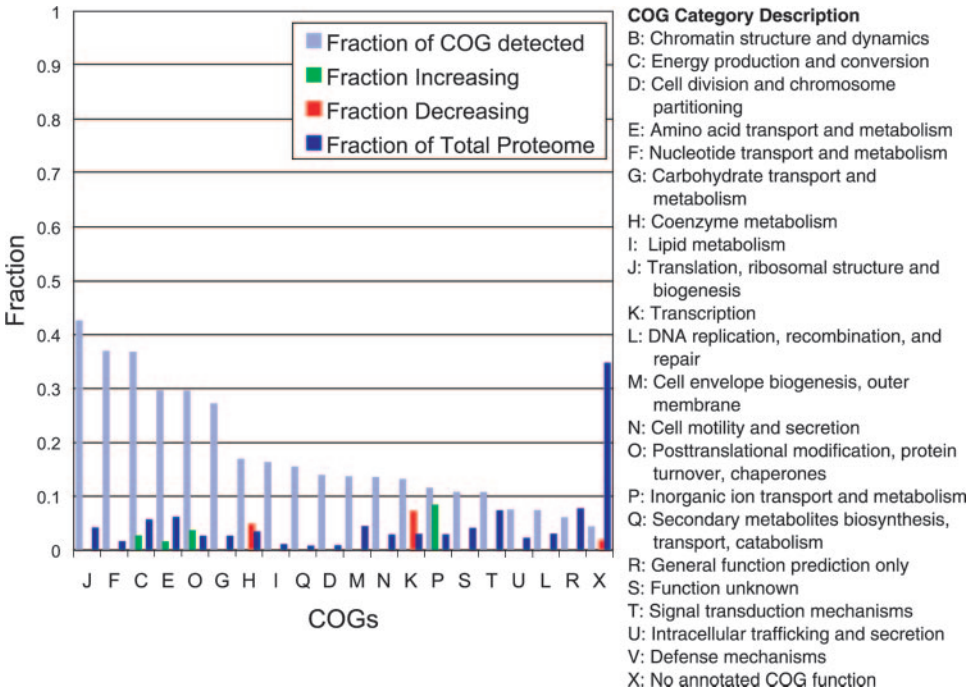

FIG. 2. Protein distribution in COGs. Proteins identified in the proteomics data cover all major COG categories (except categories B and V, which have 1 and 32 proteins, respectively). In each COG category, the fractions of protein that showed increases and decreases in the air stress experiment are indicated. The COG categories are sorted in order of decreasing fraction identified (gray bars). Notably, the largest fraction of changes was observed in COG category S (function unknown). COG categories R, L, U, and T appear to be underrepresented. Category U contains many membrane proteins, which are often not present in high abundance. The low abundance of signaling proteins may also be the reason for disproportionately low fraction of proteins in COG category T. Category X represents all proteins with no assigned COG and is the largest fraction of the total proteome, containing 1,066 proteins.

medium (39). To minimize subculturing during experimentation, *D. vulgaris* stocks stored at −80°C were used as 10% (vol/vol) inocula in 100 to 200 ml of fresh LS4D medium and the cells were grown to mid-log phase (optical density at 600 nm [OD_{600}], 0.3 to 0.4). For every transcriptome and proteome experiment, fresh starter cultures at mid-log phase were used as 10% (vol/vol) inocula in 1- to 3-liter biomass production cultures and grown at 30°C, as noted previously (39).

Cell counts and growth assays during air and 0.1% O_2 exposure. One liter of a *D. vulgaris* culture in LS4D medium at mid-log phase (OD_{600}, 0.35) was sparged with either humidified sterile N_2, 0.1% O_2 in N_2, or air (21% O_2). The sparge bottles were constructed from 2-liter medium bottles with three-valve standard high-performance liquid chromatography delivery caps (ULTRAWARE; Kimble/Kontes). One valve was used to allow gas to enter, another was used for sampling, and the third was used for gas venting. Gas was sparged through porous Teflon tubing (International Polymer Engineering, Tempe AZ) filled with glass microbeads to keep the tubing submerged in the culture. Samples were taken at 0, 60, 120, and 240 min following exposure. For measuring growth, cells were counted using the acridine orange direct count method (31). For measuring viability, CFU were determined. For these tests aliquots were taken at the time points mentioned above and diluted serially in anaerobic LS4D medium to obtain 10^2 and 10^4 dilutions. A 200-μl sample of each dilution was suspended in molten LS4D medium containing 0.8% (wt/vol) agar before it was spread on LS4D medium plates containing 1.5% (wt/vol) agar and grown anaerobically; colonies were counted after 7 days.

Biomass production for integrated "omics" experiments. Biomass for microarray analysis and proteomics experiments was generated as described previously (39). All production cultures were grown in triplicate. At an OD_{600} of 0.3 (initial time point [T_0]), triplicate samples were collected (300 ml each for microarrays and 50 ml each for proteomics). Once T_0 sampling was completed, the stress was applied by sparging humidified sterile air, 0.1% O_2 in N_2, air, or N_2 (control) at a rate of approximately 200 ml/min through the 2-liter cultures. Prior to T_0, the doubling time for *D. vulgaris* was measured to be approximately 5 h. Samples were collected at 30, 60, 120, and 240 min after sparging was initiated. Processing and chilling times were minimized by pumping samples through a metal coil immersed in an ice bath as described previously (39). The chilled samples were harvested via centrifugation, flash frozen in liquid nitrogen, and stored at −80°C until analysis. Consistent with previous studies (18), pH measurements during sparging indicated that all treatments (N_2, 0.1% O_2, or air) resulted in a small pH (<0.8-U) increase that may have been caused by H_2S and CO_2 loss during sparging. After 4 h, the pH of each culture was between 7.8 and 8.0. Using the previously reported specific oxygen-reducing potential of wild-type *D. vulgaris* (57), it could be estimated that the maximum oxygen-reducing potential of the culture was approximately 5.4 μmol O_2/min. At a sparging rate of 200 ml/min, 7.8 μmol O_2/min was estimated to be added to the culture (see Calculation S1 in the supplemental material) (http://vimss.lbl.gov/Oxygen/). Measurements with a Foxy Fospor-R oxygen sensor (Ocean Optics, Florida) indicated that continuous sparging with 0.1% O_2 increased the levels of dissolved O_2 in the blank media. The higher levels of O_2 (relative to the levels with pure N_2 sparging) were detectable in a live *D. vulgaris* culture while it was being sparged and ensured that there was constant exposure to O_2 during the 0.1% O_2 treatment (see Fig. S2 in the supplemental material; the supplemental material is also available at http://vimss.lbl.gov/Oxygen/).

Microarray transcriptomic experiments and data analysis. DNA microarrays using 70-mer oligonucleotide probes covering 3,482 of the 3,531 annotated protein-encoding sequences of the *D. vulgaris* genome were constructed as previously described (33). Briefly, all oligonucleotides were commercially synthesized without modification by MWG Biotech Inc. (High Point, NC), prepared in 50% (vol/vol) dimethyl sulfoxide (Sigma-Aldrich, St Louis, MO), and spotted onto UltraGAPS glass slides (Corning Life Sciences, Corning, NY) using a BioRobotics Microgrid II microarrayer (Genomic Solutions, Ann Arbor, MI). Each oligonucleotide probe had two replicates on a single slide. Probes were fixed onto the slides by UV cross-linking (600 mJ) according to manufacturer's protocol. Total RNA extraction, purification, and labeling were performed independently for each cell sample using previously described protocols (5). Each replicate sample consisted of cells from 300-ml cultures. Labeling of cDNA targets from purified total RNA was carried out using the reverse transcriptase reaction with random hexamer priming and the fluorophore Cy5-dUTP (Amersham Biosciences, Piscataway, NJ). Genomic DNA was extracted from *D. vulgaris* cultures

at stationary phase and labeled with the fluorophore Cy3-dUTP (Amersham Biosciences, Piscataway, NJ). To hybridize a single glass slide, the Cy5-dUTP-labeled cDNA probes obtained from stressed or unstressed cultures were mixed in equal amounts with the Cy3-dUTP-labeled genomic DNA. After washing and drying, the microarray slides were scanned using the ScanArray Express microarray analysis system (Perkin Elmer). The fluorescence intensity of both the Cy5 and Cy3 fluorophores was analyzed with the ImaGene software, version 6.0 (Biodiscovery, Marina Del Rey, CA).

Microarray data analyses were performed using gene models from NCBI. All mRNA changes were assessed with total genomic DNA as a control. Log_2 ratios and z-scores were computed as previously described (39). A mean $\log_2$ ratio cutoff of ≥ 2 across time points and an accompanying z-score of ≥ 2 were used to identify genes whose expression changed most significantly. Searches of the microarray data with the mean gene expression profile of genes in the predicted PerR regulon were performed using the Pearson correlation coefficient as the scoring function and the Euclidean distance to sort the final search results The 0.71 correlation of the rubredoxin-like protein encoded by DVU3093, the lowest-scoring gene from the predicted PerR regulon, was used as an empirical significance cutoff for the profile search results (for additional notes and analysis information see Fig. S5 in the supplemental material). All heat maps of gene expression data were rendered as vector graphics and output in Encapsulated PostScript format using JColorGrid (29). The rendering configuration specified a constant maximum and minimum data range ($\log_2$ ratio range, -6.25 to 6.25), a $\log_2$ ratio increment of 0.5, and the $\log_2$ ratio color scale centered at a $\log_2$ ratio of 0.

The specificity of transcription changes in the predicted PerR regulon genes was assessed using the mean expression of genes in the regulon computed across different experimental conditions corresponding to six previously published VIMSS studies (e.g., heat shock [5], salt stress [39], nitrite [22], and stationary phase [6]). The mean expression of genes in the PerR regulon was computed for each time point in each experiment, along with the global mean and standard deviation across all time points and experiments. To assess the confidence of the observed gene expression changes, z-scores were computed for the mean PerR gene expression at each time point in the 0.1% O_2 and air stress experiments. Assuming a normal distribution, the 95% confidence interval corresponds to a z-score of 2, and at most 5% of the data are expected to have more significant changes. In the microaerobic experiment the z-scores were 0.4, 1.2, and 1.7 for 60, 120, and 240 min, respectively. In the air stress experiment, the z-scores were -0.4, -0.8, -1.3, -1.6, and -2.5, for 0, 10, 30, 120, and 240 min, respectively. Note that this is the only calculation of z-score across multiple experiments; all other z-scores reported in this study were computed across the 0.1% O_2 and air exposure experiments only. Microarray data for this study are available at http://www.microbesonline.org/cgi-bin/microarray/viewExp.cgi?locusId=&expId=28+74. Raw microarray data can also be accessed at the following websites for 0.1% O_2 exposure and air stress: http://www.microbesonline.org/microarray/rawdata/exp28_E35 and http://www.microbesonline.org/microarray/rawdata/exp74_E12, respectively.

Proteomics and proteomics data analyses. Sample preparation, chromatography, and mass spectrometry for iTRAQ proteomics were performed as described previously (47), with modifications to the lysis buffers used. Frozen cell pellets from triplicate 50-ml cultures were thawed and pooled prior to cell lysis. For the 0.1% O_2-exposed biomass, cells were lysed via sonication in 500 mM triethylammonium bicarbonate (pH 8.5) (Sigma-Aldrich), and the clarified lysate was used as total cellular protein. Sample denaturation, reduction, blocking, digestion, and labeling with isobaric reagents were performed according to the manufacturer's directions (Applied Biosystems, Framingham, MA). The fourplex iTRAQ labels were used as follows: tag_{114}, T_0 control; tag_{115}, 240-min control; tag_{116}, 240-min 0.1% O_2-sparged sample; and tag_{117}, 240-min 0.1% O_2-sparged sample (replicate). tag_{116} and tag_{117} provided technical replicates to allow assessment of internal error. For the air-exposed biomass, cell pellets were lysed via sonication in lysis buffer (4 M urea, 500 mM triethylammonium bicarbonate; pH 8.5), and the clarified lysate was diluted with water to 1 M urea before being used. The same labeling procedure was used, and the labels were used as follows: tag_{114}, 120-min N_2-sparged control; tag_{115}, 240-min N_2-sparged control; tag_{116}, 120-min air-sparged sample; and tag_{117}, 240-min air-sparged sample. Strong cation-exchange was used to separate both 0.1% O_2- and air-exposed, iTRAQ-labeled samples into 21 to 23 salt fractions. Fractions were desalted, dried, and separated on a C_{18} reverse-phase nano-LC-MS column using a Dionex LC system coupled with an ESI-QTOF mass analyzer (QSTAR hybrid quadrupole time of flight; Applied Biosystems, Framingham, MA) as previously described (47).

Collected mass spectra were analyzed using Analyst 1.1 with ProQuant 1.1, ProGroup 1.0.6 (Applied Biosystems, Framingham, MA), and MASCOT version 2.1 (Matrix Science, Inc, Boston, MA). A FASTA file containing all the putative open reading frame sequences of *D. vulgaris*, obtained from microbesonline.org (1), was used for the theoretical search database along with the common impurities trypsin, keratin, cytochrome *c*, and bovine serum albumin. The same search parameters were used in both programs, as described previously (47). Only proteins identified by at least two unique peptides at greater than 95% confidence by both ProQuant and MASCOT were considered for further analysis.

All protein ratios were obtained from the ProQuant database using ProGroup. Tag ratios for each protein were computed as the weighted average from all peptides that were uniquely assigned to that protein. Technical replicates (tag_{116} and tag_{117} used to label 0.1% O_2-exposed biomass) were used to assess variability in quantification of $\log_2$ ratios. To define a cutoff for internal error, the deviation between the absolute values of $\log_2(tag_{116}/tag_{115})$ and $\log_2(tag_{117}/tag_{115})$ for a given protein was used. The internal error cutoff was set at the value of deviation at which 95% of all proteins showed deviation that was less than or equal to that value. The internal error cutoff was found to be 0.13 . To compute the level of significant change, a z-score was computed for all $\log_2$ values. Protein $\log_2$ values with z-scores of ≥ 2 were considered to be significantly changed. Cluster of orthologous groups (COG) categories as defined by Tatusov et al. (52) were used to plot the fraction of each COG category identified (Fig. 2). Complete proteomics data can be obtained at http://vimss.lbl.gov/Oxygen/.

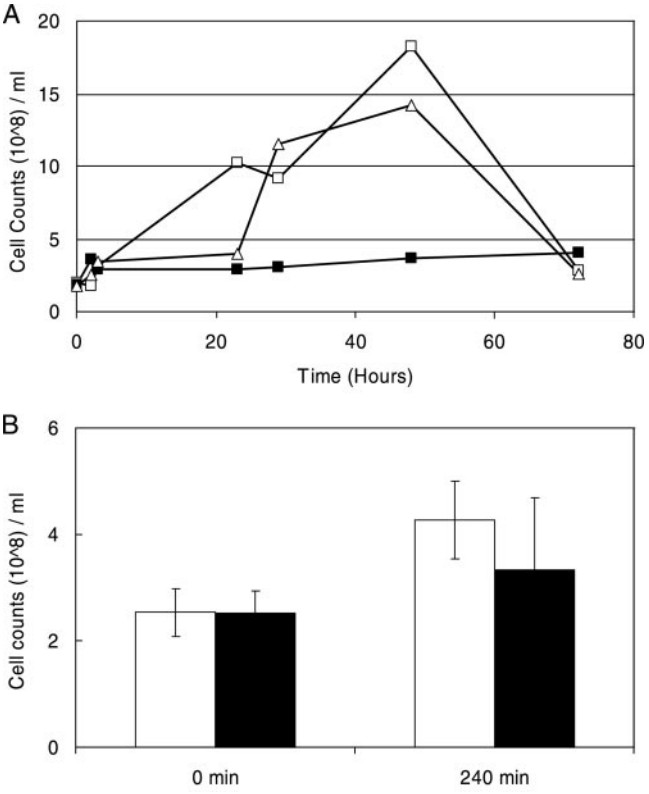

FIG. 3. Effect of O_2 exposure on growth of *D. vulgaris*. Growth of *D. vulgaris* was measured by counting the number of cells per milliliter (acridine orange direct counting). Each value is an average for three technical replicates. (A) *D. vulgaris* cell counts after sparging (200 ml/min) with 0.05% O_2 in N_2 (△), 0.1% O_2 in N_2 (■), or N_2 (□) measured over 60 h. Over the 72-h period, *D. vulgaris* showed similar growth profiles in 0.5% O_2 and N_2 (control), while in 0.1% O_2 much lower maximal growth was observed. (B) *D. vulgaris* cell counts after sparging (200 ml/min) with N_2 (open bars) or 0.1% O_2 (filled bars) at 0 and 240 min. In order to assess the cell-wide changes initiated in response to the 0.1% O_2 exposure, biomass for transcript and protein analysis was collected at 240 min after initiation of exposure, prior to entry into stationary phase. Note that the effect of 0.1% O_2 sparging is evident only at later time points.

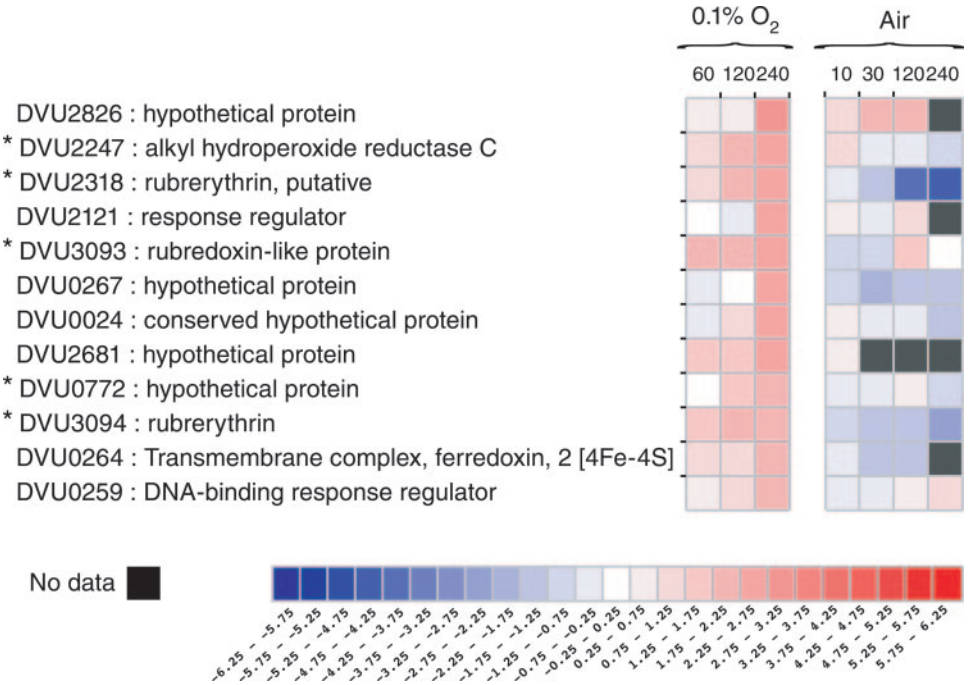

FIG. 4. Genes whose expression changed most significantly in response to 0.1% O_2 exposure (cutoff threshold, $\log_2 R \geq 2$; corresponding z-score, ≥2). The heat map shows changes in mRNA levels over time (in minutes) in response to either 0.1% O_2 or air stress. The range of changes observed for the two experiments is shown in the key below the heat map, in which the values are $\log_2 R$ values. Asterisks indicate predicted PerR regulon genes.

RESULTS

Effect of different growth conditions on biomass and viability. For genome-wide assessment of the cellular response, growth assays were conducted to determine the level of O_2 that affected the growth rate but was not lethal. Extended exposure to 0.05% O_2 had no overall effect on *D. vulgaris* growth (Fig. 3A). Consistent with this, there were no significant changes in transcript levels under these conditions (http://vimss.lbl.gov/Oxygen/). Sparging with 0.1% O_2 reduced both the growth rate and the maximal growth (Fig. 3A). However the cells resumed normal growth after a lag of about three growth cycles (15 h), and the numbers of CFU were similar to the control numbers (see Fig. S1 and S3 in the supplemental material). Therefore, 0.1% O_2 was selected as the conditions for the low-O_2-exposure experiments in this study. Although the effect of 0.1% O_2 exposure on growth was most evident at later time points, to measure the cellular response at the transcript and protein levels, biomass was collected at time points up to 240 min postexposure (Fig. 3B).

When biomass was exposed to air (21% O_2) for a similar length of time, the effect on both the growth rate and viability was drastic. Direct cell counts showed that the air-sparged samples contained only 40% of the number of cells present in the control (N_2 sparged) after 240 min of sparging. Further, measurement of the CFU indicated that only a fraction of cells formed colonies when they were plated (~10%) compared the control culture at T_0 (see Fig. S3 in the supplemental material). This result is consistent with most previous studies in which a similar reduction in viability has been documented (18); there was only one exception where CFU remained unaffected (59).

Genome-wide transcriptional response. The transcript profiles of cultures exposed to 0.1% O_2 were analyzed. Applying a $\log_2$ ratio cutoff of ≥ 2 at at least one time point (and a z-score of ≥ 2) for genes whose expression changed significantly revealed only 12 significantly upregulated genes. These results suggest that 0.1% O_2 exposure produced a mild perturbation in *D. vulgaris*. The upregulated genes included five of the six predicted members of the predicted PerR regulon (Fig. 4). Few other genes with annotated functions showed a significant change. However, *tmcB* (DVU0264) and *divK* (DVU0259) were upregulated; both of these genes belong to an operon containing an iron-sulfur cluster transmembrane ferredoxin complex. Using the same criteria, no transcript showed significant downregulation.

It is noteworthy that following exposure to 0.1% O_2, expression of the *perR* transcript increased with time, as did the expression of the transcripts of all other predicted PerR regulon genes (Fig. 4). In addition to the predicted PerR regulon, the *D. vulgaris* genome contains many genes thought to protect against oxidative damage that are widely present across many classes of bacteria, including genes encoding superoxide dismutase (*sodB*), catalase (*kat*), and several thioredoxins (Fig. 5). Based on conservation across SRB, several oxidative stress response genes are considered to be signature genes in SRB (5), and they include genes encoding predicted oxygen response candidates, such as the Sor operon and genes encoding several ferritins (Fig. 6). Of the genes encoding functions inferred to protect against oxidative damage, neither the genes widely distributed nor the signature genes showed a significant transcript change in response to 0.1% O_2 exposure. Microarray

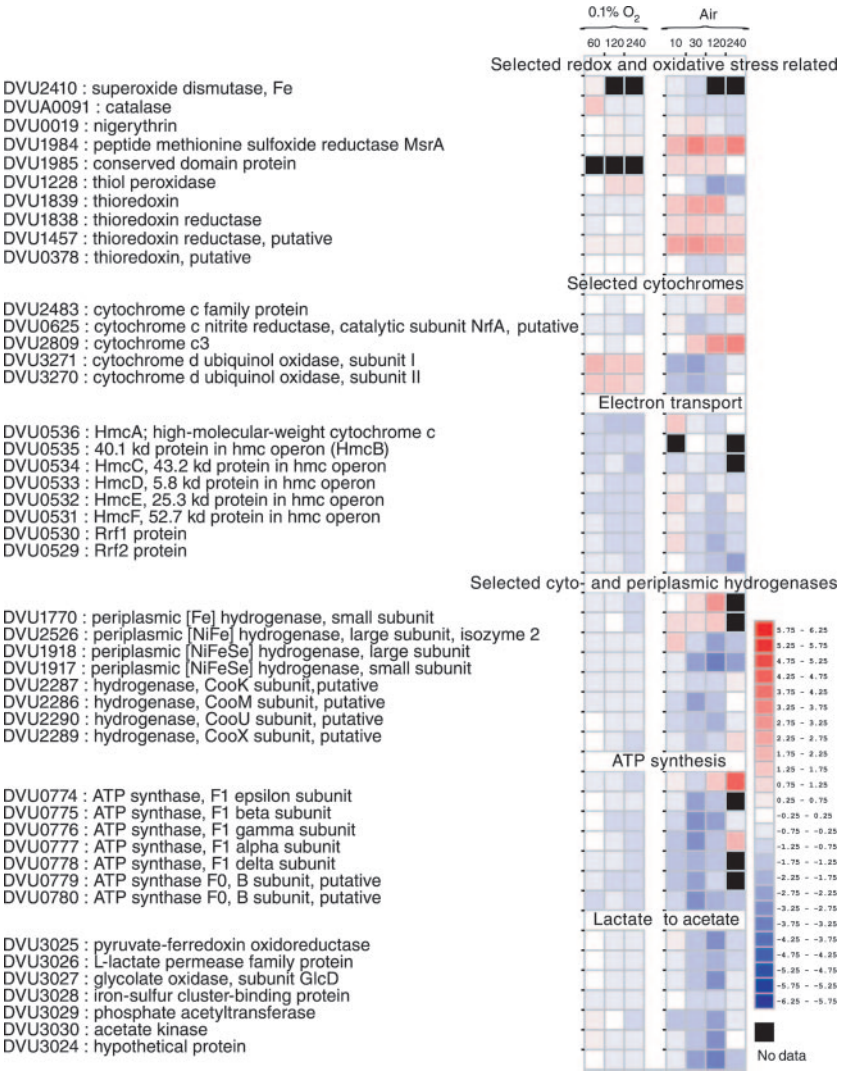

FIG. 5. Transcriptomic responses of selected genes in 0.1% O_2- and air-exposed cultures. The heat map shows changes in mRNA levels over time (in minutes) in response to either 0.1% O_2 or air stress. Candidates are grouped by function or gene identification numbers, and groups are not from automated clustering. The range of changes observed for the two experiments is shown in the key to the right of the heat map. The candidates included are genes considered important in redox changes and genes for central pathways, such as electron transport, ATP synthesis, carbon uptake, and metabolism.

data also indicated that genes encoding proteins predicted to be involved in central metabolic pathways, such as the sulfate reduction pathway, ATP synthesis, and several periplasmic or cytoplasmic hydrogenases, were unaffected during 0.1% O_2 exposure (Fig. 5 and 6).

In contrast, air exposure generated a large number of differentially expressed genes; 393 candidates showed significant upregulation, whereas 454 genes were found to be downregulated (for complete data see the microarray data website provided in Materials and Methods). Among these, genes in the predicted PerR regulon were downregulated, as were signature SRB genes and other genes considered to provide protection from oxidative stress (Fig. 4, 5, and 6). Further, in contrast to the response in the 0.1% O_2 exposure experiment, significant downregulation of many genes in central pathways was recorded in the air exposure experiment (Fig. 5 and 6), highlighting the striking difference in the *D. vulgaris* responses to the two conditions. The upregulated transcripts in the air-stressed biomass included genes encoding *clp* proteases, chaperone proteins, and phage shock proteins (Fig. 7), which was suggestive of a drastic stress response. None of these genes showed any change during exposure to 0.1% O_2.

Proteomic response. An iTRAQ proteomics strategy was used to identify differences in protein content for the same samples used for the microarray analysis. A total of 251 proteins were identified by two independent mass spectrometry analysis software packages (see Materials and Methods) (47). As in the microarray data, proteins were considered to be significantly changed if their absolute z-scores were >2. Responses at the protein level may lag those at the transcript level, and this may account for the milder proteomic changes compared to the microarray results. The greatest change noted was more than twofold ($\log_2$ ratio, 1.37). For z-scores of ≥ 2 there were only four proteins with increased levels and two

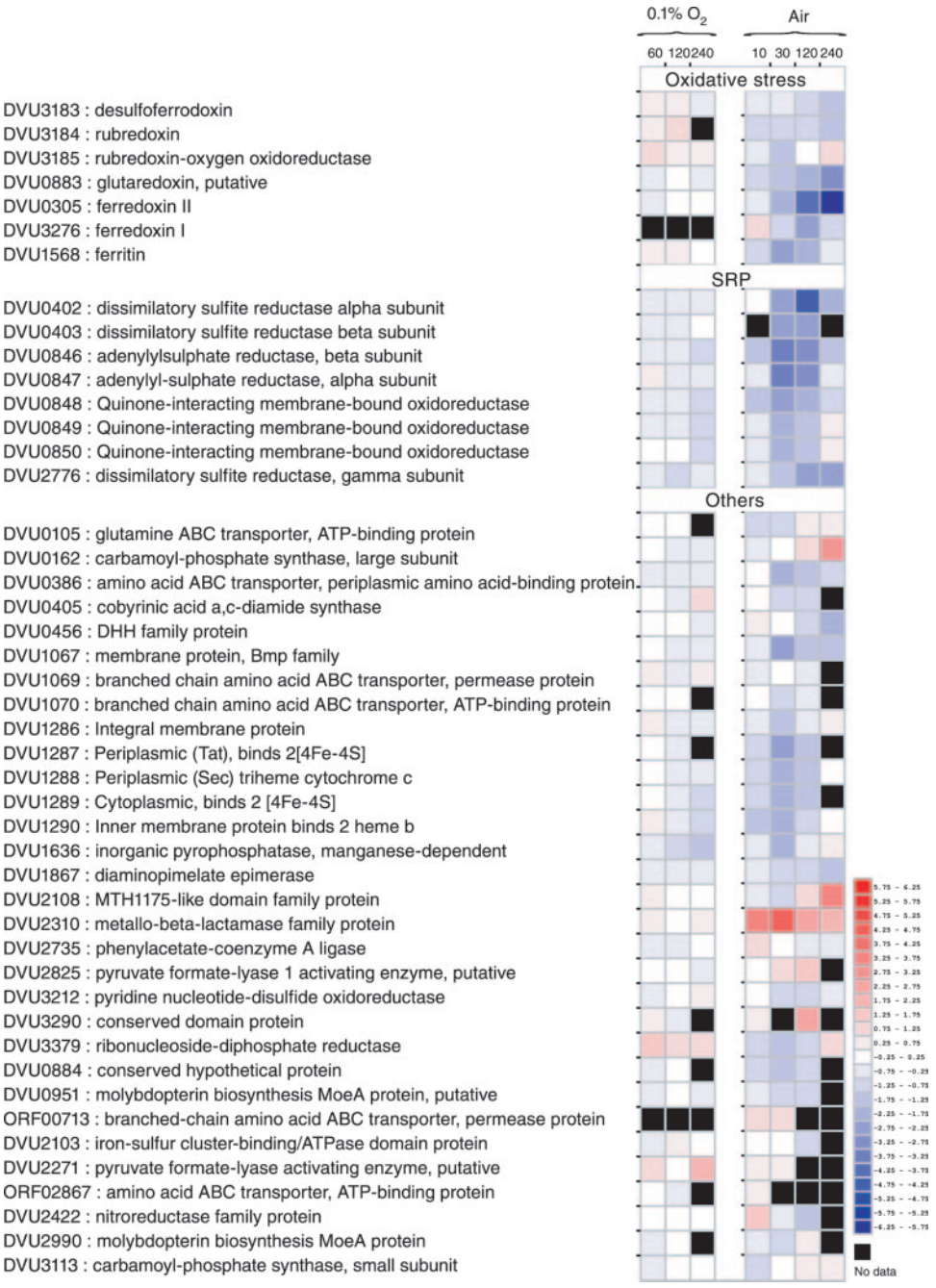

FIG. 6. Transcriptomic responses of signature SRB genes during 0.1% O_2 and air exposure. The heat map shows changes in mRNA levels over time (in minutes) in response to either 0.1% O_2 or air stress. Signature genes described by Chhabra et al. (5) were used. Genes are categorized by function. The range of changes observed for the two experiments is shown in the key to the right of the heat map.

proteins with decreased levels. Three of the six predicted PerR regulon members were identified in the proteomics data, and all were present at higher levels in the 0.1% O_2-exposed biomass (Fig. 8 and Table 1). Proteins for other oxygen response mechanisms, such as Sod (DVU2410), RoO (DVU3185), and proteins encoded by the Sor operon were also identified, but no significant changes were observed. The only other protein that showed accumulation with 0.1% O_2 exposure was a putative zinc resistance-associated protein, ZraP (DVU3384), although the mRNA levels did not reflect this change. Only two proteins, Rho (DVU1571), a predicted transcription termination factor, and IlvE (DVU3197), a predicted branched-chain amino acid aminotransferase, showed decreased levels. While many proteins involved in central metabolism (e.g., ATP synthesis, sulfate reduction, and pyruvate-to-acetate conversion) were identified, none of these proteins showed any significant change in response to 0.1% O_2 exposure, consistent with the microarray data.

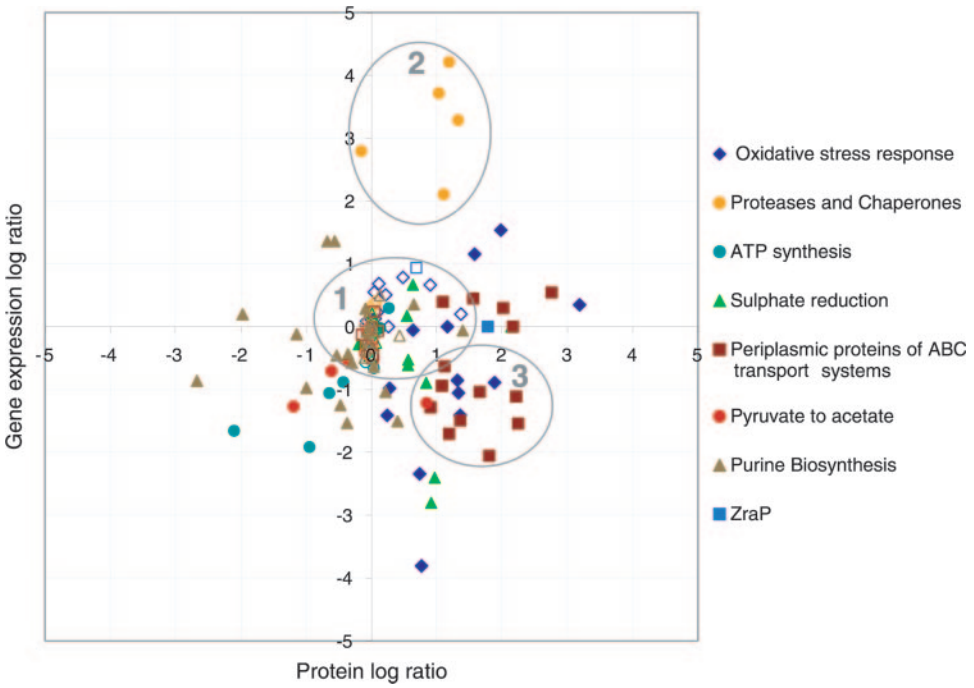

FIG. 7. Comparison between proteomics and microarray data for selected candidates: graphical representation of data presented in Table 1. The open symbols represent 0.1% O_2 exposure, whereas the filled symbols represent air exposure. Circle 1 highlights all of the candidates belonging to the low-oxygen-exposure group. The most significant changes occurred in oxidative stress genes and in ZraP. Air exposure caused a much larger level of change. Circle 2 highlights the large increases observed in proteases and chaperones during air exposure. Circle 3 highlights the group of periplasmic binding ABC transport proteins that show opposite trends, namely, increased protein levels but decreased transcript levels. More candidates show this trend, compared to the few candidates that show increased transcript levels but decreased protein levels (top left quadrant).

Proteomics analysis of air-stressed biomass was conducted at both 120 min and 240 min. As shown in Fig. 8C, the response at 120 min showed a trend similar to that at 240 min. A total of 438 proteins were identified in this analysis. Thirty-three proteins exhibited a significant change after 120 min of air sparging, while 16 changed following 240 min of air sparging (Table 1). In contrast to the 0.1% O_2 exposure, in air stress conditions Sod (DVU2410) showed the largest increase, and this increase was confirmed by immunoblotting (see Fig. S4 in the supplemental material). The proteomics data from the air-stressed biomass also identified proteins in most central pathways (Table 1); however, no concerted significant changes could be seen across any pathways. Notably, neither the open reading frame annotated as ZraP nor the predicted PerR regulon showed any significant change at 240 min during air exposure.

PerR regulon expression profile. The genes of the predicted PerR regulon showed a distinct expression pattern with both 0.1% O_2 exposure and aerobic stress across several time points (Fig. 9). The mean expression profile for the predicted PerR regulon genes was used to search the remainder of the microarray data for other transcripts showing similar changes. Many transcripts correlated with the mean expression profile of the predicted PerR regulon genes across the two conditions and sets of time points. Among the genes of the predicted PerR regulon, the gene most correlated to that of the mean PerR profile was the rubrerythrin gene (DVU3094; correlation, 0.98), and the least correlated was a rubredoxin-like protein gene (DVU3093; correlation, 0.71). Using 0.71 as an empirical score significance cutoff, the PerR mean expression profile search identified 58 candidates. As evidence of the specificity of the information contained in the mean PerR expression profile, we analyzed the score distribution of the PerR regulon members in the search results. The top five of six candidates from the search were five of six members of the PerR regulon: a rubrerythrin gene (DVU3094) (Pearson rank/final rank, 1/2; correlation, 0.98), *ahpC* (Pearson rank/final rank, 2/1; correlation, 0.95), the PerR gene (Pearson rank/final rank, 3/6; correlation, 0.94), a hypothetical protein gene (DVU0772) (Pearson rank/final rank, 5/1; correlation, 0.89), and a putative rubrerythrin gene (DVU2318) (Pearson rank/final rank, 6/58; correlation, 0.89) (Fig. 9) (see Fig. S5 and Table S1 in the supplemental material). Six of eight transcripts in the predicted *tmc* operon, encoding the tetraheme cytochrome c_3 complex, also showed high correlation with the PerR profile: DVU0260 (correlation, 0.83), DVU0265 (0.83), DVU0267 (0.82), DVU0264 (0.80), DVU0266 (0.77), and DVU0263 (0.75). The *cydA* and *cydB* genes that encode the putative cytochrome *bd* oxidase were also correlated with the mean PerR regulon gene expression profile, at 0.74 and 0.69 for *cydA* and *cydB*, respectively (however, *cydB* was correlated below the level of the empirical correlation cutoff). The remaining genes in the top matches of the profile search were genes encoding 10 conserved hypothetical proteins and 37 hypothetical proteins (see Table S1 in the supplemental material).

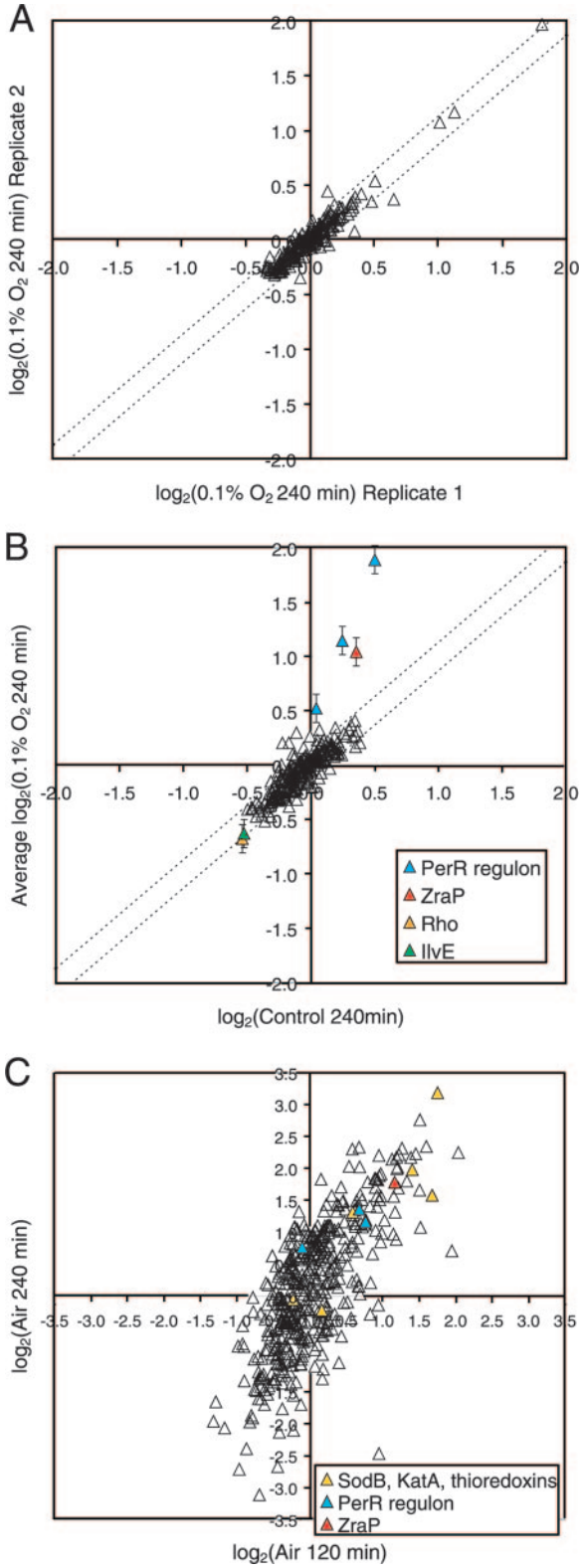

FIG. 8. iTRAQ proteomics for exposure to 0.1% O_2 and air. (A) The 0.1% O_2-exposed sample was labeled with both tag_{116} (replicate 1) and tag_{117} (replicate 2), allowing assessment of the internal error. (B) Log_2 (0.1% O_2/T_0) compared to log_2 (N_2/T_0). Proteins whose z-score was ≥ 2 were considered significant, and these candidates are highlighted, as indicated in the key. (C) Log_2 (air/N_2) at 120 min compared to the log_2 (air/N_2) at 240 min. Proteins that have the same level of change at both time points would fall on the 45° line. The clustering of data around the 45° line demonstrated that there was a trend in changes observed between 120 min and 240 min. Selected proteins are color coded as indicated in the key.

DISCUSSION

While continuous bubbling of the *D. vulgaris* culture with 0.1% O_2 ensured cell exposure to a proportional amount of O_2, this level of O_2 exposure produced only a mild perturbation. This finding is reflected in the small number of genes that changed expression and the fact that no changes were observed in central metabolic genes. This may be an indication that under normal growth conditions, *D. vulgaris* already contains adequate levels of most of the enzymes required to respond to low levels of O_2 exposure. Concerted upregulation of the entire predicted PerR regulon was observed during 0.1% O_2 exposure, and *ahpC* was one of the most upregulated candidates at both the transcript and protein levels. Along with the *tmc* transmembrane cytochrome c_3 operon response, these were the only cellular responses to 0.1% O_2 exposure. PerR regulons have been described in many bacteria (3, 21, 24, 25, 48, 58), and genes regulated by PerR are often involved in defense against ROS accumulation. In *D. vulgaris*, predicted members of the PerR regulon, such as a rubrerythrin (DVU0265), have been identified as important enzymes during exposure to both O_2 and other oxidative stresses (18).

The air stress had a much more drastic effect on a cell-wide level. The responses at the mRNA level were reproducible across biological replicates (Fig. 10B). Further, the changes in transcript levels for air-stressed biomass at 120 and 240 min were self-consistent, having a Pearson correlation of 0.77. The proteomics measurements for the biomass were similarly self-consistent, having a Pearson correlation of 0.73 (Fig. 8B). The microarray data indicated that there was overall downregulation in central metabolic pathways such as sulfate reduction, ATP synthesis, electron transfer, lactate uptake, and conversion of lactate to acetate, none of which were observed with 0.1% O_2 exposure. The downregulation of genes such as lactate permease and lactate dehydrogenase genes during air exposure may be representative of cellular stress or a defensive response to prevent use of the electron donor and consequently prevent reduction of oxygen. Most importantly, upon air exposure the transcript levels for the predicted PerR regulon genes decreased overall, and transcripts for *perR* and genes encoding rubrerythrin and the putative rubrerythrin decreased consistently with time and showed 4-fold to 24-fold downregulation. These results highlight a sharp contrast in the responses of *D. vulgaris* to 0.1% O_2 exposure and air exposure.

Using the mean expression profile for the predicted PerR regulon genes across the two exposures, the microarray data were searched for other transcripts with similar expression profiles. The resulting list contained several members of the eight-gene operon encoding the transmembrane tetraheme cytochrome c_3 complex (DVU0258 to DVU0266) and also the *cydAB* operon (DVU3270 and DVU3271) encoding the cytochrome *d* ubiquinol oxidase proteins. The cytochrome *bd* oxidase system is typically involved in oxidative phosphorylation,

TABLE 1. Selected proteomics data

DVU no.	Gene name	Description	iTRAQ $\log_2$ (0.1% O_2/control) at 240 min ($\pm$0.13)[a,b]	Microarray $\log_2$ (0.1% O_2/control) at 240 min[b]	iTRAQ $\log_2$ (air/control) at 240 min ($\pm$0.13)[a,b]	Microarray $\log_2$ (air/control) at 240 min[b]
Oxidative stress response proteins						
DVU0995		ThiJ/PfpI family protein		0.42 (0.8)	1.33 (1.16)	−1.05 (−1.89)
DVU1228	*tpX*	Thiol peroxidase	0.16 (0.77)	0.5 (0.84)	0.24 (0.09)	−1.41 (−2.54)
DVU1397	*bfr*	Bacterioferritin	−0.02 (0.05)	0.12 (0.22)	0.28 (0.13)	−0.98 (−1.18)
DVU1457	*trxB*	Thioredoxin reductase, putative	0.15 (0.75)	0.11 (0.22)	1.98 (1.79)	1.53 (2.75)
DVU1568	*ftn*	Ferritin	−0.13 (−0.38)	0.22 (0.38)	0.74 (0.58)	−2.34 (−4.3)
DVU1839	*trx*	Thioredoxin	0.14 (0.7)	−0.1 (−0.18)	1.58 (1.4)	1.15 (1.84)
DVU2247	*ahpC*	Alkyl hydroperoxide reductase C	1.89 (7.41)	0.19 (0.35)	1.36 (1.18)	−1.4 (0)
DVU2318	*rbr2*	Rubrerythrin, putative	1.14 (4.56)	0.66 (1.13)	0.77 (0.6)	−3.8 (0)
DVU2410	*sodB*	Superoxide dismutase, Fe	0.32 (1.4)		3.19 (2.97)	0.34 (0)
DVU3049		Hemerythrin family protein	0.27 (1.19)	0.08 (0.14)	1.88 (1.7)	−0.89 (−1.41)
DVU3094	*rbr*	Rubrerythrin	0.52 (2.16)	0.78 (1.36)	1.16 (0.99)	
DVU3183	*sor*	Superoxide reductase		0.59 (1.09)	1.69 (1.51)	2.31 (1.69)
DVU3185	*roo*	Rubredoxin-oxygen oxidoreductase	0 (0.11)	0.54 (1.03)	0.64 (0.48)	−0.05 (−0.1)
DVUA0091	*kat*	Catalase	0.19 (0.9)	0.68 (0.84)	1.32 (1.14)	−0.85 (−1.25)
Proteases, chaperones, and other stress responses						
DVU0811	*dnaK*	DnaK	0.03 (0.26)	0.24 (0.39)	1.03 (0.87)	3.28 (5.62)
DVU1012		Hemolysin-type calcium-binding repeat	−0.39 (−1.37)	0.3 (0.45)	1.19 (1.02)	3.71 (0)
DVU1468	*htrA*	Peptidase/PDZ domain	−0.36 (−1.27)	0.39 (0.74)	1.11 (0.94)	4.21 (6.74)
DVU1976	*groEL*	Chaperonin, 60 kDa	−0.35 (−1.21)	0.34 (0.47)	−0.16 (−0.3)	2.1 (3.82)
DVU1977	*groES*	Chaperonin, 10 kDa		0.15 (0.25)	1.33 (1.15)	2.79 (3.63)
DVU3384	*zraP*	Zinc resistance-associated protein	1.04 (4.16)	0.93 (1.24)	1.78 (1.6)	
Periplasmic proteins of ABC transport systems						
DVU0095	*potD-1*	Polyamine ABC transporter, periplasmic polyamine binding		−0.5 (−0.84)	1.56 (1.38)	0.44 (0.81)
DVU0107	*glnH*	Glutamine ABC transporter, periplasmic glutamine binding		−0.1 (−0.19)	2.25 (2.05)	−1.54 (−2.74)
DVU0169		Oligopeptide/dipeptide ABC transporter, periplasmic	0.09 (0.49)	−0.2 (−0.3)	2.22 (2.02)	−1.11 (−1.54)
DVU0386	*glnH*	Amino acid ABC transporter, periplasmic	0.12 (0.62)	−0.44 (−0.77)	1.36 (1.18)	−1.49 (−2.82)
DVU0547		High-affinity branched-chain amino acid ABC transporter, periplasmic	0.04 (0.3)	−0.29 (−0.51)	1.19 (1.02)	−1.7 (−3.33)
DVU0675	*fliY*	Amino acid ABC transporter, periplasmic	0.22 (0.97)		2.17 (1.97)	
DVU0712		Amino acid ABC transporter, periplasmic binding	0.25 (1.14)	−0.07 (−0.14)	1.08 (0.91)	−0.94 (0)
DVU0752		Amino acid ABC transporter	−0.28 (−0.97)	−0.3 (−0.55)	1.1 (0.92)	0.39 (0.7)
DVU0966		Amino acid ABC transporter, periplasmic	−0.14 (−0.41)	−0.5 (−0.92)	1.8 (1.62)	−2.05 (−3.6)
DVU1238		Amino acid ABC transporter, periplasmic		−0.3 (−0.59)	1.66 (1.48)	−1.04 (−1.96)
DVU1937		Phosphonate ABC transporter, periplasmic	−0.04 (−0.02)	−0.06 (−0.12)	0.91 (0.74)	−1.28 (−2.3)
DVU2297		Glycine/betaine/L-proline ABC transporter, periplasmic binding	0 (0.12)	0.23 (0.38)	2.02 (1.83)	0.29 (0.55)
DVU2342		Amino acid ABC transporter, periplasmic		−0.51 (−0.93)	1.12 (0.95)	−0.63 (−1.03)
DVU3162		ABC transporter, periplasmic substrate-binding protein	0.09 (0.51)	−0.13 (−0.24)	2.76 (2.55)	0.54 (0)
ATP synthesis						
DVU0775	*atpD*	ATP synthase, F1 beta subunit	−0.21 (−0.68)	−0.39 (−0.63)	0.27 (0.11)	0.29 (0.35)
DVU0777	*atpA*	ATP synthase, F1 alpha subunit	−0.13 (−0.38)	−0.57 (−0.97)	0.13 (−0.01)	−0.02 (−0.02)
DVU0778	*atpH*	ATP synthase, F1 delta subunit	−0.24 (−0.78)	−0.66 (−0.94)	−0.43 (−0.57)	−0.88 (−1.42)
DVU0114	*hisG*	ATP phosphoribosyltransferase		−0.2 (−0.21)	−2.1 (−2.2)	−1.65 (−3.21)
DVU0779	*atpF2*	ATP synthase F0, B subunit		−0.89 (−1.56)	−0.64 (−0.78)	−1.06 (−1.17)
DVU0780	*atpF1*	ATP synthase F0, B subunit		−0.39 (−0.69)	−0.95 (−1.07)	−1.91 (−2.97)
Sulfate reduction						
DVU0402	*dsrA*	Dissimilatory sulfite reductase, alpha subunit	0.05 (0.35)	0.09 (0.17)	0.97 (0.8)	−2.4 (−2.49)
DVU0403	*dvsB*	Dissimilatory sulfite reductase, beta subunit	0.22 (1)	0.01 (0.01)	0.91 (0.75)	−2.8 (−4.74)

Continued on following page

TABLE 1—*Continued*

DVU no.	Gene name	Description	iTRAQ $\log_2$ (0.1% O_2/control) at 240 min (±0.13)[a,b]	Microarray $\log_2$ (0.1% O_2/control) at 240 min[b]	iTRAQ $\log_2$ (air/control) at 240 min (±0.13)[a,b]	Microarray $\log_2$ (air/control) at 240 min[b]
DVU0404	*dsrD*	Dissimilatory sulfite reductase D	−0.02 (0.04)	0.2 (0.3)	2.14 (1.95)	
DVU0847	*apsA*	Adenylyl-sulfate reductase, alpha subunit	0.02 (0.25)	−0.08 (−0.13)	0.84 (0.67)	−0.89 (−1.32)
DVU0848	*qmoA*	Quinone-interacting membrane-bound oxidoreductase	−0.03 (0)	−0.5 (−0.89)	0.56 (0.4)	−0.61 (−1)
DVU0849	*qmoB*	Quinone-interacting membrane-bound oxidoreductase	−0.1 (−0.27)	−0.46 (−0.67)	0.54 (0.39)	0.16 (0.26)
DVU1295	*sat*	Sulfate adenylyltransferase	0.07 (0.42)	−0.08 (−0.14)	−0.19 (−0.34)	−0.28 (0)
DVU1597	*sir*	Sulfite reductase, assimilatory type	−0.37 (−1.3)	−0.05 (−0.1)	0.63 (0.47)	0.66 (1.22)
DVU2776	*dsrC*	Dissimilatory sulfite reductase, gamma subunit	−0.23 (−0.76)	−0.25 (−0.44)	0.56 (0.41)	−0.52 (−0.57)
Purine biosynthesis						
DVU0161	*purF*	Amidophosphoribosyltransferase		−0.22 (−0.37)	−0.36 (−0.5)	−0.43 (−0.72)
DVU0488	*purD*	Phosphoribosylamine-glycine ligase	0.31 (1.37)	−0.06 (−0.08)	−0.56 (−0.7)	1.36 (0)
DVU0795	*purC*	Phosphoribosylaminoimidazole-succinocarboxamide synthase	−0.02 (0.03)	−0.34 (−0.52)	−0.09 (−0.23)	0.28 (0.45)
DVU1043	*guaA*	GMP synthase		0.02 (0.03)	−0.68 (−0.81)	1.35 (2.62)
DVU1044	*guaB*	IMP dehydrogenase	−0.21 (−0.7)	0.09 (0.15)	−1.15 (−1.26)	−0.12 (−0.21)
DVU1406	*purM*	Phosphoribosylformylglycinamidine cyclo-ligase		−0.33 (−0.63)	−0.48 (−0.61)	−1.25 (−1.65)
DVU1932	*adk*	Adenylate kinase (TIGR)	0.11 (0.57)	−0.49 (−0.68)	0.4 (0.24)	−1.5 (−2.97)
DVU2942	*purB*	Adenylosuccinate lyase	0.2 (0.93)	−0.14 (−0.24)	0.65 (0.49)	0.35 (0.53)
DVU3181	*purL*	Phosphoribosylformylglycinamidine synthase II	0.04 (0.32)	−0.18 (−0.28)	−1.97 (−2.07)	0.19 (0.34)
DVU3204	*purA*	Adenylosuccinate synthetase	0 (0.11)		0.04 (−0.11)	−0.64 (−1.16)
DVU3206	*purH*	Phosphoribosylaminoimidazolecarboxamide formyltransferase		0.28 (0.5)	−0.37 (−0.51)	−1.53 (−3.02)
DVU3235	*purH*	IMP cyclohydrolase, putative	0.26 (1.17)	0.06 (0.11)	1.4 (1.22)	−0.06 (−0.11)
Pyruvate to acetate						
DVU3025	*por*	Pyruvate-ferredoxin oxidoreductase	−0.3 (−1.03)	−0.04 (−0.07)	−0.36 (−0.5)	−0.56 (−0.8)
DVU3027	*glcD*	Glycolate oxidase, subunit	−0.23 (−0.75)	−0.42 (−0.77)	−0.61 (−0.74)	−0.7 (−0.81)
DVU3029	*pta*	Phosphate acetyltransferase	−0.29 (−1)	−0.47 (−0.81)	−1.19 (−1.31)	−1.27 (0)
DVU3030	*ackA*	Acetate kinase	0 (0.16)	−0.31 (−0.54)	0.85 (0.68)	−1.21 (−1.36)

[a] ±0.13 represents the internal error cutoff computed as described in Materials and Methods.
[b] The values are $\log_2$ ratios, and the values in parentheses are the corresponding z-scores; only values for which the z-score is ≥2 were considered a significant change.

and increases in the transcription of the corresponding genes during oxidative stress have been reported for other anaerobic bacteria, such as *Desulfovibrio gigas* (38), *Moorella thermoacetica* (11), and *Bacteroides fragilis* (2). These enzymes also appear to have a protective role in aerobic bacteria such as *Escherichia coli* and *Salmonella* during oxidative stress (15, 34). The existence of cytochrome *bd* oxidases in *D. vulgaris* has been a matter of historical discussion since pure cultures of *D. vulgaris* are unable to grow in oxygen (8). Here, the significant increase observed in transcripts for the electron transfer systems such as the *tmc* cytochrome c_3 complex and for the oxidative phosphorylation enzymes like cytochrome *bd* oxidase may indicate that additional copies of these enzymes play a protective role during 0.1% O_2 exposure.

Several redox-active proteins, such as a thiol peroxidase, bacterioferritin, flavodoxin, and ferredoxins, also correlated with the mean PerR regulon gene expression profile. Since the levels of these candidates also increased during 0.1% O_2 exposure, they may also be required for defense against O_2 in *D. vulgaris*. Other oxidative response genes, including the rubredoxin gene (DVU3184), present in the Sor operon and the Sor gene itself were also identified by the gene expression profile search, but no significant upregulation of these candidates was observed. Of the 58 candidates, more than one-third (21) have no predicted functions. Among the genes for which a functional annotation exists, several chemotaxis and signal transduction genes were identified. These genes are ideal candidates for further study to confirm any specific role in the oxidative stress response.

It has recently been demonstrated that a *roo* deletion strain of *D. vulgaris* was more sensitive to microaerobic stress than the wild type (57); however, we observed no change in expression of this gene at either the transcript or protein level in the 0.1% O_2 exposure experiments. Deletion of the genes encoding Sor and Sod has been shown to create strains with greater O_2 sensitivity (18). While neither of these genes showed a significant transcriptional change during 0.1% O_2 exposure, candidates that confer fitness and ensure survival may already be present and not necessarily show changes in transcript or protein levels. Compared to 0.1% O_2 exposure, exposure to air appears to have a severely detrimental effect on cellular growth. It should be noted, however, that increases in the Sod protein levels and the few additional upregulated transcripts in oxidative stress response genes (such as the genes encoding

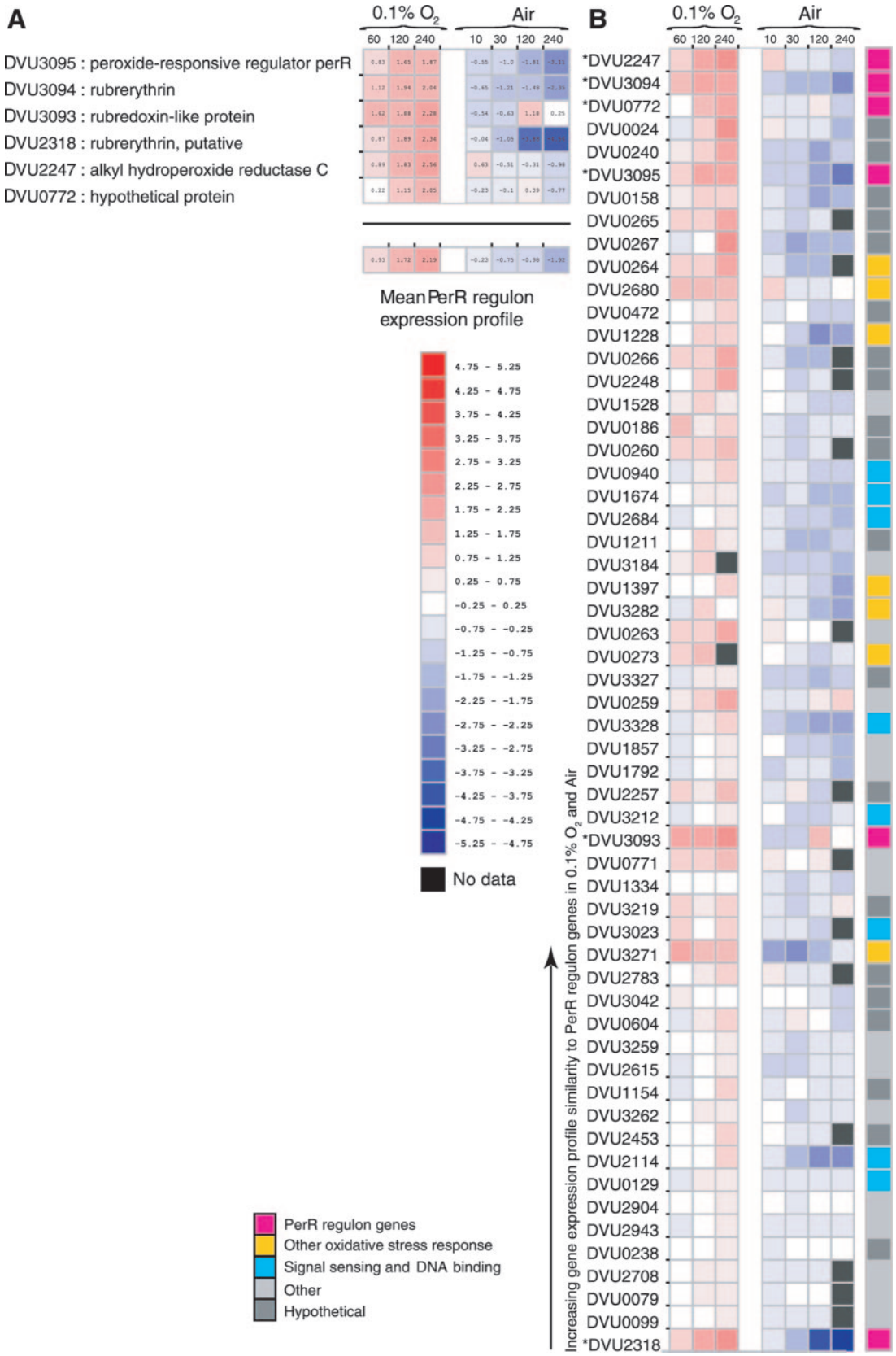

FIG. 9. Analysis of microarray data to extract genes that show changes correlated with changes in the predicted PerR regulon. (A) Heat map showing changes in mRNA levels for the predicted members of the PerR regulon with 0.1% O_2 and air exposure. The average trend for each time point for all the members is shown in the bottom panel. The average values from panel A were used to search the entire data set. A Pearson correlation similarity measure showed 58 genes with a trend better than or equal to that of the worst-fitting member of the PerR regulon (see Fig. S4 in the supplemental material). (B) Heat map for mRNA changes for the 58 genes. The color key indicates the predicted functional categories of these genes. For complete details of this list and the Pearson correlation function used, see Table S1 in the supplemental material.

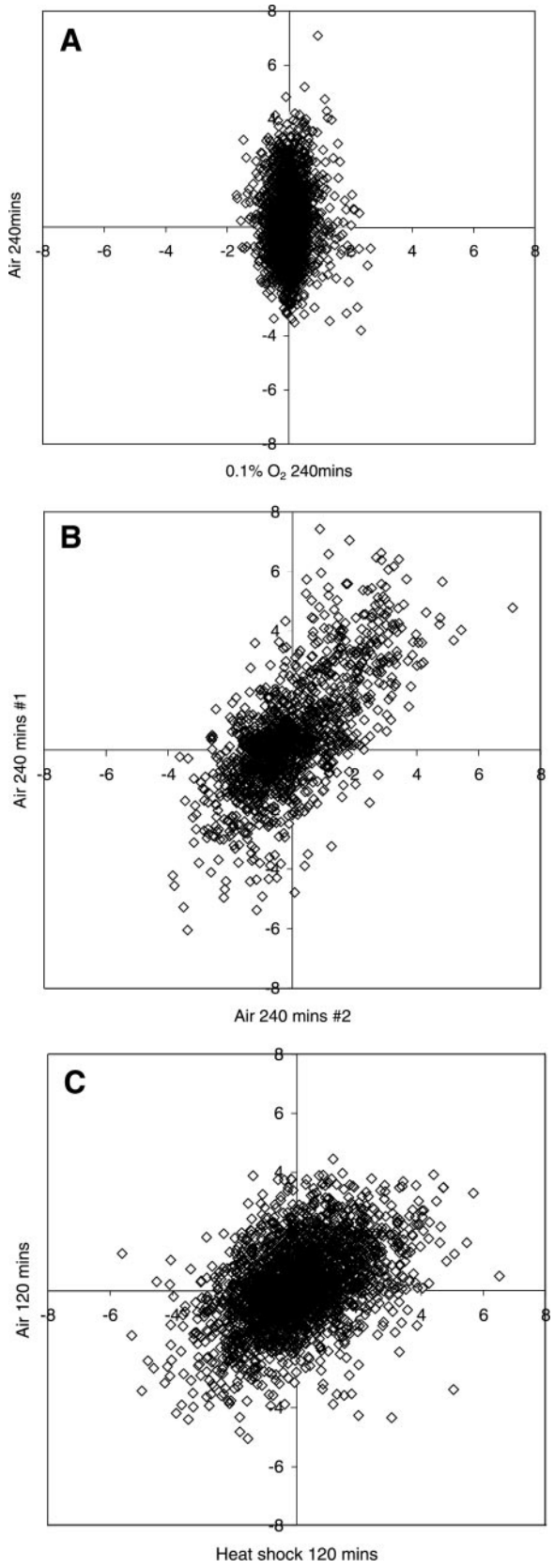

FIG. 10. Microarray data for air stress. (A) Comparison of mRNA data for exposure to 0.1% O_2 and for exposure to air shows no linear relationship (Pearson correlation coefficient, 0.03; $P = 0.01805$). (B) Comparison mRNA data of two biological replicates exposed to air at 240 min. Although exposure to air created a heterogeneous population, the responses of two different biological replicates correlate strongly (Pearson correlation coefficient, 0.69; $P < 0.000005$). The data for the second biological replicate are data from an independent experiment. (C) Heat shock (50°C, 120 min) data from the study of Chhabra et al. (5) compared with the 120-min air exposure data. Direct comparisons of these data were possible because both experiments used the same microarray design, the biomass samples came from the same pipeline, and the microarray experiments used genomic DNA as a control. A stronger linear relationship exists between the overall trends observed for heat shock versus air exposure (Pearson correlation coefficient, 0.45; $P < 0.000005$). All P values are based on the one-tailed t-statistic.

putative peptide methionine sulfoxide reductases, *msrA* and *msrB* [DVU0576 and DVU1984]) in the air-stressed biomass may be physiologically relevant for the small population of cells that remain viable during air exposure.

Genes in the predicted PerR regulon have exhibited perturbations in other *D. vulgaris* functional genomics studies (e.g., studies of heat shock [5], salt stress [39], nitrite stress [22], and stationary phase [6]). An increase in all members of this predicted regulon was also seen with heat shock (5), but the time-dependent increase shown by these genes appears to be unique to 0.1% O_2 exposure. Additionally, while a large number of upregulated genes were documented in the heat shock study, the upregulation during 0.1% O_2 exposure of the predicted PerR regulon genes constitutes a much more specific and limited transcriptional response. Based on all the data, it appears that PerR derepression is the primary *D. vulgaris* response to low-O_2 exposure. Interestingly, the air stress transcriptomic data correlated better with heat shock data than with the data from 0.1% O_2 exposure (Fig. 10), and the predicted PerR-regulated genes were significantly downregulated with air stress, further supporting the specificity of PerR derepression during low-O_2 exposure. The common changes for air stress and heat shock have also been noted in a previous study (59).

Another candidate that was universally upregulated across multiple stress conditions monitored in *D. vulgaris* was a protein annotated as zinc resistance-associated protein ZraP (DVU3384). Although it was highly upregulated in both conditions studied here, DVU3384 may be a general stress response candidate. Additionally, although zinc uptake regulons have been shown to increase with O_2 exposure in lactobacilli (50) and with oxidative stress in *Bacillus* (19), the DVU3384 protein may not be a zinc binding protein. In proteins with confirmed zinc binding motifs, such as *E. coli* YjaI, known to preferentially bind Zn and Ni (43), Zn binding is conferred by a two-part motif: an N-terminally located sequence, HRWH GRC, and a C-terminally located sequence, HGGHGMW. Due to the evolutionary distances between this gammaproteobacterium and the deltaproteobacterium sulfate reducer and the low sequence similarity to experimentally validated proteins, more experimental proof is required to confirm the metal ion binding specificity of the *D. vulgaris* ZraP (DVU3384). However, the *D. vulgaris* ZraP sequence contains a cysteine residue in the C-terminal region, as well as multiple histidine residues in the N-terminal region, both contained in

glycine-rich and presumably flexible regions of the protein. Together, these data suggest that *D. vulgaris* ZraP contains a likely metal binding site and is an interesting candidate for follow-up experiments.

Many bacteria traditionally categorized as anaerobic organisms, including *Helicobacter pylori* (56) and *Bacteroides fragilis* (2), contain numerous mechanisms to counter O_2 stress. Other anaerobes, such as *Clostridium* spp., *M. thermoacetica*, and *Spirillum winogradskii* (4, 10, 11, 32, 44), have also been found to tolerate transient exposure to oxic environments. While some of these organisms are microaerophilic, *D. vulgaris*, like *H. pylori* and *Clostridium* spp., cannot utilize O_2 for growth and is anaerobic by definition. However, our data indicate that this bacterium can survive 0.1% O_2 exposure both in terms of growth and in terms of cellular response and appears to be entirely suited for ecological niches that experience transient exposure to O_2. Results from previous studies have shown that the members of the Sor operon and other oxidative stress response genes are important for the survival of *D. vulgaris* during O_2 exposure (18, 55). Our study suggests that additional protection may be provided by the peroxidases in the predicted PerR regulon and membrane-bound cytochromes. The very concerted increase and temporal response of the predicted PerR regulon in *D. vulgaris* upon exposure to low concentrations of oxygen is consistent with a physiological response to a condition that may be frequently encountered in the natural environment. Seasonal episodic infiltration of snow melts and rainfall events bring oxygenated waters to previously established anoxic and reducing environments. Given the ability of *D. vulgaris* to cope with low O_2 levels for short periods, these weather-related effects are unlikely to be catastrophic. Further, despite the graver consequences of exposure to higher levels of O_2, even the limited viability ensures propagation of the bacterium through this exceedingly harsh stress. This further suggests why *D. vulgaris* and other SRB are so resilient in a variety of habitats, including those where exposure to oxygen may occur periodically.

ACKNOWLEDGMENTS

We are grateful to Morgan Price, Eric Alm, and Katherine Huang for helpful discussions. We thank Rick Huang, Richard Phan, and Mary Singer for technical help with biomass production, Barbara Giles for sharing experimental observations, and Keith Keller and Janet Jacobsen for help with the supplemental materials link.

This work was part of the Environmental Stress Pathway Project (ESPP) of the Virtual Institute for Microbial Stress and Survival (http://vimss.lbl.gov) supported by the U.S. Department of Energy Office of Science Office of Biological and Environmental Research Genomics: GTL Program through contract DE-AC02-05CH11231 with Lawrence Berkeley National Laboratory. Oak Ridge National Laboratory is managed by University of Tennessee-Battelle LLC for the Department of Energy under contract DE-AC05-00OR22725.

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