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Title

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Abstract

Desulfovibrio vulgaris was cultivated in a defined medium and biomass was sampled over time for approximately 70 h to characterize the shifts in gene expression as cells transitioned from exponential to stationary phase growth during electron donor depletion. In addition to temporal transcriptomics; total protein, carbohydrate, lactate, acetate, and sulfate levels were measured. The microarray data were analyzed for statistically significant expression changes, hierarchical cluster analysis, and promoter element prediction, and were validated via quantitative PCR. As the cells transitioned from exponential to stationary-phase a majority of the down-expressed genes were involved in translation and transcription, and this trend continued in the remaining time points. Intracellular trafficking and secretion, ion transport, and coenzyme metabolism showed a general increase in relative expression as the cells entered stationary phase. As expected, DNA replication machinery was down expressed, and genes involved in DNA repair increased in expression during stationary-phase. Genes involved in amino acid acquisition, carbohydrate metabolism, energy production, and cell envelope biogenesis did not exhibit uniform transcriptional responses. Interestingly, most phage-related genes were up-expressed at the onset of stationary-phase. This result suggested that nutrient depletion may impact community dynamics and DNA transfer mechanisms of sulfate-reducing bacteria via phage cycle. The putative *feo*AB system (in addition to other presumptive iron metabolism genes) was significantly up-expressed, and suggested the possible importance of Fe^{2+} acquisition under metal-reducing conditions. A large subset of carbohydrate-related genes had altered gene expression, and the total cellular carbohydrate levels declined during the growth phase transition. Interestingly, the *D. vulgaris* genome does not contain a putative *rpo*S gene, a common attribute of the δ-*Proteobacteria* genomes sequenced to date, and other putative *rpo* genes did not have

significantly altered transcription profiles. Our results indicated that in addition to expected changes (e.g., energy conversion, protein turnover, translation, transcription, and DNA replication/repair) genes related to phage, stress response, carbohydrate flux, outer envelop, and iron homeostasis played important roles as *D. vulgaris* cells experienced electron donor depletion.

Introduction

The underground corrosion of metal pipes used for gas or water and the generation of sulfide during digestion of domestic and agricultural wastes have been the economic and environmental processes that have historically driven the desire to understand the metabolism of the SRB (sulfate-reducing bacteria) (16). The SRB have been a particular problem for the petroleum industry not only because of their role in metal corrosion but also due to petroleum souring and health hazards of the hydrogen sulfide. In contrast, SRBs can be advantageous for bioremediation processes. A variety of studies (6, 15, 17, 33, 39) have documented the ability of SRB, including *Desulfovibrio* spp., to reduce toxic metals such as U(VI) and Cr(VI) enzymatically, a process that results in the production of less water-soluble species. The modification of solubility properties caused by changing the redox state of the metal presents itself as a potential avenue for bioremediation of contaminated groundwater and soils. Previous research specifically points toward SRB as environmentally-relevant experimental systems for the study of heavy metal and radionuclide reduction (2, 3, 32). Sulfate-reducers provide several advantages with respect to heavy-metal reduction including the presence of sulfate in a variety of environments and the protection of immobilized heavy metals from oxidation with iron sulfides (mackinawite) (4).

D. vulgaris Hildenborough has been studied extensively and much is already known about the metabolic versatility of this bacterium (21, 53). *D. vulgaris* is capable of coupling the oxidation of a variety of electron donors (e.g., lactate, pyruvate, succinate, ethanol) to the reduction of many different electron acceptors (e.g. sulfate, fumarate, uranium, chromium, potentially O_2) either directly or concomitantly. To effectively immobilize heavy metals and radionuclides, it is important to understand the cellular responses to adverse factors observed at contaminated subsurface environments, such as mixed contaminants and the changing ratios of electron donors and acceptors. As documented in other bacteria, the cellular response to different stressors (e.g., osmolyte, heat, pH) can overlap with responses to stationary phase growth (23). In addition, the stimulation of microbial populations with carbon and/or energy sources to promote bioremediation can impact the physiology of indigenous microorganisms in relation to the available electron acceptors. The identification of stasis-induced genes and gene networks will provide fundamental information about the cellular processes needed for survival under pertinent field conditions (e.g., low-nutrients, slow-growth, energy source variability), and a comparative framework for additional stressors and environmental stimuli. The above studies demonstrate that the study of *Desulfovibrio* spp. with respect to sulfate- and metal-reduction is well founded, and that the developed genomic and proteomic tools in *D. vulgaris* provide the resources to gain an in-depth understanding of cellular processes important for stress responses and bioremediation in relation to environmental conditions. In this study we focus on stasis-induced genes and gene networks by looking at transition of *D. vulgaris* from exponential- to stationary-phase. Our results demonstrated that *D. vulgaris* cells altered gene expression profiles in response to carbon and energy depletion, and that the expression of genes during stationary-phase was not static.

Materials and Methods

Bacterial strains and growth conditions. *Desulfovibrio vulgaris* ATCC 29579 was grown in a batch system that consisted of a glass column (diameter, 7 cm) with a diffuser, a septum, and a gas outlet. The columns contained LS4D medium (1 l) that was gassed with nitrogen at an approximate rate of 1 ml/min and constantly stirred. LS4D is a defined medium with approximately 50 mM Na_2SO_4, 60 mM $NaC_3H_5O_3$, 8.0 mM $MgCl_2$, 20 mM NH_4Cl, 2.2 mM K_2HPO_4/KH_2PO_4 (pH 7.2), 0.6 mM $CaCl_2$, Thauers vitamins (9), trace minerals (9), 30 mM PIPES buffer, 0.06 μM resazurin, and 10 mM NaOH to pH to 7.2. Triplicate columns were inoculated with a 10% (v/v) inoculum from the same culture of *D. vulgaris* to an approximate optical density (600 nm) of 0.06. Cultures were incubated in a 30°C water bath and samples were collected aseptically at various time points via a septum.

Sample collection. During growth, triplicate cultures were sampled at 9 different time points for RNA extraction and global expression analyses. Sample time points were selected based upon monitored protein and lactate levels, and represented exponential phase (T1 to T3), transition (T4 and T5), and stationary phase (T6 to T9). Samples (approximately 5 mg protein) for RNA extraction were collected at various time points throughout the growth phases. Samples were collected into sterile, cold (-20°C) beakers and were then immediately pumped through a metal coil (ID, 3 mm) kept at -20°C ($CaCl_2:H_2O$ slurry) to chill the sample. After the sample was quickly cooled and collected, samples were centrifuged for 6 min at 4,000 x g (4°C). The supernatant was discarded and the pelleted sample was then submerged into liquid nitrogen to snap freeze. The pellets were stored at -70°C until used for RNA extraction. Samples (1 to 5 ml) were also collected for the determination of protein and total carbohydrate associated with cell pellets, and supernatants were used for the determination of lactate, sulfate, and acetate levels. Samples were stored at -20°C until processing.

Chemical analyses. Protein was analyzed with the Lowry method (34) and bovine serum albumin (Pierce Biochemicals) as a standard. Carbohydrate was determined with a cysteine-sulfuric acid colorimetric assay as previously described (10). Lactate, acetate, and sulfate concentrations were measured via ion chromatography (Metrohm-Peak) with a Metrosep organic acid column and a Metrosep Anion Supp 5 column, respectively. All assays were done in duplicate and the variation was less than 10%. The results for each column were averaged to provide a complete protein and carbohydrate profile.

Oligonucleotide probe design and microarray construction. DNA microarrays covering 3,482 of the 3,531 annotated protein-coding sequences of the *D. vulgaris* genome were constructed with 70mer oligonucleotide probes as previously described (18, 19, 20). Following the examination of the entire probe set according to the oligonucleotide probe design criteria (7), 3,471 (97.1%) specific oligonucleotide probes were obtained, and 103 (2.9%) remained non-specific. In addition, 10 oligonucleotides for 10 human genes and 10 oligonucleotides for 10 *Arabidopsis* genes were selected against the *D. vulgaris* genome for positive (with mRNA spiked) or negative (without mRNA spiked) controls. All designed oligonucleotides were commercially synthesized without modification by MWG Biotech Inc., (High Point, NC). The concentration of oligonucleotides was adjusted to 100 pmol/µl in 50% DMSO (v/v) and were 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 duplicates on a single slide. In addition, 6 different concentrations (5, 25, 50, 100, 200, and 300 ng/µl) of genomic DNA were spotted (8 duplicates on a single slide) as positive controls. After printing, the oligonucleotide probes were fixed onto the slides by UV cross-linking (600 mJ of energy) according to the manufacturer's protocol (Corning Life Science, Corning. NY).

Total RNA extraction, purification and labeling. Total cellular RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. RNA extracts were purified according to the RNeasy Mini Kit (Qiagen Valencia, CA) instructions and on-column DNase digestion was performed with RNase-free DNase (Qiagen, Valencia, CA) to remove genomic DNA.

To generate cDNA probes with reverse transcriptase, 10 μg of purified total RNA was used for each labeling reaction as previously described (20, 50). Briefly, random hexamers (Invitrogen) were used for priming and the fluorophor Cy3-dUTP or Cy5-dUTP (Amersham Biosciences, Piscataway, NJ) was used for labeling. After the labeling, RNA was removed by NaOH treatment and cDNA was immediately purified with a Qiagen PCR Mini kit. The efficiency of labeling was routinely monitored by measuring the absorbance at 260 nm (for DNA concentration), 550 nm (for Cy3), or 650 nm (for Cy5). Two samples of each total RNA preparation were labeled, one with Cy3-dUTP and another with Cy5-dUTP for microarray hybridization.

Microarray hybridization, washing and scanning. For determination of the overall hybridization signals, sensitivity, and the number of detected genes, a genomic DNA or RNA sample labeled with a single dye was used as previously described (20). Hybridization was conducted using a slide for each biological replicate and each gene had duplicate probes. The microarrays were hybridized at 45°C overnight with 50% formamide. The labeled cDNAs were re-suspended in 20 to 25 μl of hybridization solution that contained 50% formamide (v/v), 1 mM DTT, 3x saline-sodium citrate (SSC), 0.3% SDS (w/v), and 0.8 μg/μl of herring sperm DNA (Invitrogen Life Technologies, CA). The sample was incubated at 98°C for 5 min, centrifuged to collect condensation, and kept at 50 to 60°C. The sample was immediately applied onto the

microarray slide, covered with cover slip, and hybridization was carried out in a waterproof Corning hybridization chamber (Corning Life Science, NY) submerged in a 45°C water bath in the dark for 16 h. After hybridization, cover slips were immediately removed and washed in a buffer containing 1 x SSC and 0.1% SDS (w/v) for 5 min at 37°C. Microarrays were washed in a new buffer with 0.1 x SSC and 0.1% SDS (w/v) for another 5 min at room temperature. Finally, microarrays were washed with distilled water for 30 sec at room temperature and dried by compressed air or by centrifugation. Microarrays were scanned using a ScanArray 5000 microarray analysis system (Packard BioChip Technologies, MA). Typically, 95-100% of laser power and 70 to 80% of PMT were selected for scanning.

Image quantification and data analysis. To determine signal fluorescence intensities for each spot, 16-bit TIFF scanned images were analyzed by application of the software ImaGene version 6.0 (Biodiscovery, Marina Del Rey, CA) to quantify spot signal, spot quality, and background fluorescent intensities. Empty spots, poor spots, and negative spots (e.g., spots without deposited probe) were flagged according to the instruction of the software and removed in subsequent analysis (14).

The resulting data files were subjected to Lowess intensity-based normalization and further analyzed using GeneSpring version 5.1 (Silicon Genetics, Redwood City, CA). To assess the statistical significance of individual data points, the Student t-test was used to calculate a p-value to test the null hypothesis that the expression level was unchanged. A statistical model incorporating both per gene variance (z values) and operon structure was further used to compute the posterior probability that each gene changed its expression level in the direction indicated by its mean value (40). Members of operons without a consistent signal across replicates were excluded, and genes with Log_2 Ratios of $|Z|<2.0$ were considered significant. The first time point

was used as the control sample, and all subsequent samples were compared to T1 in order to elucidate gene expression changes during transition to stationary-phase growth.

Hierarchical Cluster Analysis. Microarray data sets from different time points were analyzed with average linkage hierarchical cluster analysis with Euclidean distance matrices and visualized with TreeView as previously described (13, 47). If a gene had expression data for all time points, and an expression level of $\log_2$ (Ratio) $\geq$ 1.5 or $\leq$ -1.5 at one or more time points, this gene was used for clustering analysis, where Ratio = gene expression at one of time points (T2 to T9)/gene expression at T1. A gene expression of $\log_2$ (Ratio) $\geq$ 1.5 was considered as up-expression, and a gene expression of $\log_2$ (Ratio) $\leq$ -1.5 was considered as down-expression.

Quantitative PCR. Quantitative PCR was done on RNA extracts from samples taken at 5, 18, 23, 29, 35, 43, 51h. Total RNA (5 µg) was used to prepare cDNA by reverse transcription. The RNA sample was mixed with 6 µg of random primers (Invitrogen) and the total volume brought to 21 ul with DNase/RNase-free water. The mixture was incubated at 70°C for 10 min and then placed on ice to cool. After cooling, 4 µl of 5X reverse transcription buffer, 2 µl of 0.1M DTT, 1 µl of 10 mM dNTPs, 1 µl of RNase Out inhibitor (Invitrogen), and 1 µl of reverse transcriptase (Invitrogen) were added to each tube. The tubes were incubated at 42°C for 2 h and then incubated at 70°C for 15 min. The tubes were cooled on ice and absorbance (OD_{260}) was determined. The cDNA samples were diluted to a concentration of 25 ng/µl and a 2 µl sample of cDNA was used for quantitative PCR (qPCR).

Primers used for qPCR are described in Table 2. The primers were diluted to a 10 mM working stock. The qPCR reaction mixture contained 10 µl of SYBR Green Master Mix (Applied Biosystems), 0.7 µl each of 10 mM forward and reverse primer, 2 µl of cDNA template, and 6.6 µl of nuclease-free water to achieve a final volume of 20 µl. The real-time

PCR reaction was carried out in a Rotor-gene 6 (Corbett Research, Sydney, Australia) with the following conditions: 1 cycle of 95°C for 10min, 40 cycles of 95°C for 15 s, 55°C for 30 s, and 60°C for 30 s. Standards were made from genomic DNA with a starting concentration of 10^8 copies/µl. A serial dilution was done to obtain a standard curve of 10^8 to 10^1 copies/µl, and a negative control that did not contain RTase was done on all samples. When real-time-PCR analysis was used to quantify the expression levels of nine genes, a high degree of correlation was observed between results from microarrays and real time-PCR (R=0.88), as reported in previous studies that used similar microarray procedures and techniques (11, 14, 18, 54).

Promoter element prediction. Gibbs Recursive Sampler (31, 52) was used to predict transcription factor-binding motifs upstream of 136 (119 from chromosome and 17 from megaplasmid) differentially expressed *D. vulgaris* genes unique to stationary phase (from T5 to T9) over T1 as described previously (54). The Gibbs Recursive Sampler program was used with (i) 16-bp models allowed to fragment up to 24 bp; (ii) up to three sites per sequence, where $P(0$ sites$) = 0.2$, $P(1$ site$) = 0.7$, $P(2$ sites$) = 0.05$, and $P(3$ sites$) = 0.05$; (iii) a position-specific background model to account for variation in local base composition; and (iv) the Wilcoxon signed-rank test (http://bayesweb.wadsworth.org/gibbs/gibbs.html). The sequences used for alignment were intergenic regions ≥ 25 bp upstream of putative translation start codons, as defined by the *D. vulgaris* genome annotation (NC_002937 and NC_005863). Negative controls for the Wilcoxon test were randomly chosen from the set of *D. vulgaris* intergenic regions, provided that the sequence lengths matched those of the test sequences and excluded any sequence region that included a promoter for a gene that showed at least a twofold change in expression during stationary phase of *D. vulgaris* over T_1. The Wilcoxon test calculates a *P* value for the motif, given the null hypothesis that the sequences under study (the test sequences)

are not more likely to contain sites than the negative control sequences and also consider the score ranks of the predicted sites in the motif. The program SCAN was used to identify sites from a database that were described by the motif (35). SCAN calculates a P value for each of the identified sites that reflect the probability that a site with that score or better would occur in a random database of the same size.

Results

Growth. The three replicate cultures were grown in a defined medium with lactate and sulfate in batch-mode, and the inoculum was in exponential-phase growth. Optical density (OD_{600}) was used to monitor growth for the determination of sampling points. The initial biomass level was approximately 20 µg/ml protein, and reached approximately 260 µg/ml protein in 25 h (Fig. 1).

Total carbohydrate levels increased steadily during exponential phase, and plateaued as the cells entered stationary-phase (Fig. 1). These results suggested that the exhaustion of electron donor also caused a shift in the carbon flux of the cells. The total carbohydrate levels declined during stationary-phase growth and were 2- to 3-fold lower at the last sampling. Our preliminary results have shown that the majority of measured carbohydrate in planktonic cells is internal (Fields and Clark, unpublished). These results suggested that the measured carbohydrate was glycogen, and previous results have documented glycogen in *Desulfovibrio* spp. (44).

The exponential-phase growth rate was 0.11 h^{-1} and the generation time was 6.41 h, and the cessation of exponential growth coincided approximately with the depletion of lactate (Fig. 2). Sulfate and lactate was depleted concomitantly, lactate declined at a faster rate, and the decline in sulfate levels ceased upon depletion of lactate (Fig. 2). The pH steadily increased over the course of growth, and reached a pH value of approximately 9.0 in early stationary-phase growth.

Transcriptomics. During growth, triplicate cultures were sampled 9 times for RNA extraction and global expression analyses, four times in exponential-phase and five times for stationary-phase growth (Fig. 1). The sampling time points are indicated in Fig. 1. The samples in exponential-phase growth (T2 and T3) had few genes significantly altered in expression when compared to T1, and a minimal number of ORFs displayed significant changes in transcription even at T4 (Fig. 3). At T5 through T9, approximately 130 to 250 presumptive ORFs were up-expressed as the cells experienced electron donor depletion, and 90 to 130 ORFs were down-expressed (Fig. 3). In addition, many hypothetical proteins (HP) and conserved hypothetical proteins (CHP) had altered gene expression for most of the time points.

When the significant changers were categorized into COGs, several of the groups were predominantly represented by up- and down-expressed ORFs, respectively (Fig. 4a and b). At 20 h and 23 h, genes predicted to be involved in amino acid transport/metabolism, energy production/conversion, and translation were the dominant groups that were down-expressed. These three groups accounted for the largest fraction of down-expressed genes for all time points, in addition to transcription and signal transduction (Fig. 4b).

At 23 h (late-exponential), approximately half of the down-expressed genes were involved in translation and transcription, and this trend continued in the remaining time points. Genes involved in amino acid acquisition, carbohydrate metabolism, energy production, and cell envelope biogenesis showed both up- and down-expression (Fig. 4a and b), and these results suggested that subsets of genes in the same COG may be specific to a particular growth phase.

Six small-subunit (SSU) and 10 large-subunit (LSU) rRNA protein genes were down-expressed greater than 2-fold between 20 and 30 h post-inoculation, but the levels of expression appeared to rebound once stationary-phase was established albeit at lower levels than observed

for exponential-phase growth (Suppl. Fig. 1). Other presumptive genes associated with translation were also down-expressed and included: nusG, EF-Ts, and IF-1. Other proteins known to be involved in nutrient deprivation are SsrA and SmpB in *Escherichia coli* (26). The expression level of the *smp*B homolog in *D. vulgaris* did not change greater than 2-fold when compared to T1, but the levels did change over time. Finally, the Era protein of *E. coli* has recently been shown to specifically bind to SSU rRNA and the 30S ribosomal subunit and is thought to be a cell-cycle check point for protein synthesis (45). DVU0052 annotated as a G-protein similar to Era was drastically up-expressed at the 29 h sample point.

Hierarchical cluster analysis of time points. The sampling points were sorted into clusters based upon similar patterns of expression across the entire genome ($\geq$1.5 z values). When the sampling points were compared to the first time point (6 h), T2 (14 h), T3 (20 h), and T4 (23 h) were clustered (Fig. 5). However, the sampling points later in exponential phase growth (20 and 23 h) were more closely grouped. These results suggested that cells were beginning to alter gene expression profiles 5 h before depletion of lactate, and this observation coincided with the down-expression of at least 14 ribosomal proteins well before the lactate was completely depleted (Suppl. Fig. 1). Based upon cluster analysis, several genes were preferentially up-expressed at 20 and 23 h and included genes coding for: a putative hemerythrin-cation binding protein (DVU0170), a putative TonB protein (DVU2390), a putative ABC-transporter (DVU2380), and a conserved hypothetical protein (DVU0273). Interestingly, DVU0170 and DVU2390 were predicted to be involved in metal (i.e., Fe) transport, and DVU0273 and DVU2380 were predicted to have a FUR binding box upstream of the predicted translational start site (43). Later during the time-course, other putative proteins involved in iron

homeostasis displayed significant changes in expression (discussed below), and these proteins could be part of a first response to iron limitation as lactate was depleted.

The cluster analysis indicated that the gene expression profile significantly shifted between the 23 and 29 h samplings (Fig. 5), and represents the transition between exponential and stationary phases. The second major cluster contained the later time points (29, 35, 43, 51 and 67 h), but the 67 h (the last time point) time point was dissimilar from the other stationary-phase time points (Fig. 5). In addition, a distinction could be observed between the cells at 29 h and collectively at 35 to 51 h. The results indicated that transcriptional expression patterns were indicative of growth phase transitions, and that distinct profiles could be observed for relative early- (approximately 5 h post-exponential), mid-(approximately 10 to 30 h post-exponential), and late-stationary-phases (approximately 40 h post-exponential).

A group of 33 genes were preferentially up-expressed at 29 h, and could be placed into 4 major categories: lipoproteins, amino acid metabolism, metal-binding, and phage-related. Most phage related genes were up-expressed at 29 h and remained up-expressed during the reminder of the experiment (representatives in Suppl. Fig. 2). A group of five lipoproteins (DVU0163, 1366, 2367, 2428, 2496) were up-expressed and at least two were predicted to be in the outer membrane. Genes predicted to be involved in amino acid metabolism (DVU0601, 1012, 1331, 1413, 2297, 3293) had more similar expression levels at 29 h than other time points. Two ORFs that may be involved in iron acquisition were preferentially up-expressed at 29 h, and were a rubrerythrin with a ferritin-like domain (DVU0019) and a hemerythrin-cation binding protein (DVU0170).

The 35, 43, and 51 h time points were grouped, and most likely represented 'mid-stationary' phase during the course of the experiment (Fig. 5). Genes that had similar levels of

expression during these time points could be categorized into the following groups: HPs/CHPs, phage-related genes, DNA-binding proteins, and control of translation. Three ORFs predicted to be involved in translation were DVU0084, an alternative initiation factor that recycles GTP/GDP, DVU2218, a putative EngC that is predicted to have a role in translation regulation, and DVU2486, a putative acetyltransferase that acts upon ribosomal proteins.

Iron acquisition. With respect to possible iron acquisition as the cells survived during carbon and energy-source depletion, three ORFs predicted to be part of a Fe^{2+}-transport system (DVU2571, 2572, 2574) were up-expressed at 20 h, peaked at 23 h, and then transcription declined after 29 h (Suppl. Fig. 3). The putative *feo* system is comprised of FeoAB, and a similar system is involved in Fe^{2+} transport in *E. coli* (25). Concurrently, nigerythrin (DVU0019) was up-expressed at 29 h, but then expression declined past the 29 h sampling. The *D. vulgaris* genome has nine ORFs annotated to have ferritin domains, and most were down-expressed or displayed little change during the growth conditions. At the 67 h sampling, the cells had experienced 42 h of electron donor depletion. Related to possible iron acquisition, two genes that were preferentially up-expressed at 67 h were DVU0103 and DVU3170. DVU0103 is a putative *fep*C that is predicted to be an ABC-type cobalamin/Fe(III)-siderophore transport system, and DVU3170 is predicted to be a precorrin-3B C17-methyltransferase involved in cobalamin biosynthesis.

Key genes for lactate and sulfate utilization. *D. vulgaris* has six putative lactate permease genes (DVU2110, 2285, 2451, 2683, 3026, and 3284), and lactate is a main carbon and energy source for the *Desulfovibrio* genus under sulfate-reducing conditions. Two permeases were unchanged, three were down-expressed, and one was up-expressed (Suppl. Fig. 4). The

permeases changed expression levels between 14 and 23 h, and this time point represented when the lactate was half the initial levels to almost complete exhaustion.

The *D. vulgaris* genome has three ORFs annotated as sulfate permeases (DVU0053, 1999, 0279), and each displayed a different expression trend as cells transitioned from exponential to stationary phase. DVU0053 increased in levels from 14 to 29 h, and then decreased throughout stationary phase. DVU0279 was the inverse of DVU0053, and decreased from 14 to 29 h and then remained relatively low. DVU1999 was at relatively low levels, but gradually increased over the course of the experiment (Suppl. Fig. 5). It is important to note that sulfate levels decreased initially up to the 29 h sample, and then remained constant around 15 mM throughout stasis (Fig. 2).

Three ORFs were annotated as putative lactate dehydrogenase (LDH) genes (DVU0600, 2784, 0253) in the *D. vulgaris* genome. Two displayed a trend of elevated levels during exponential growth; however; DVU0253 increased at a faster rate and decreased significantly after 23 h. DVU0600 transcripts increased at a slower rate during exponential phase growth and did not show a down-regulation until after the 29 h sample point (data not shown). The ORF, DVU2784, remained mostly unchanged. The expression of the putative *dsr*A and *dvs*B genes (dissimilatory sulfite reductase subunits) remained largely unchanged, but did show slight down-expression over time starting at the transition to stationary-phase (data not shown).

Representative genes associated with stasis-induced stress. *D. vulgaris* has 11 ORFs predicted to encode proteins classified as universal stress proteins, based on relatedness to the Usp proteins of *E. coli* (29). Previous research in *E. coli* has shown that UspA protein levels can become elevated in response to starvation for carbon, nitrogen, phosphate, sulfate, and amino acids as well as exposure to heat, oxidants, metals, and antibiotics (29). All the *D. vulgaris*

putative sequences were classified as class I UspA-related proteins. Three ORFs (DVU0006, 0452, 0893) were relatively stable throughout exponential growth, but displayed a down-expression between the 23 and 29 h sampling (Suppl. Fig. 6a). In contrast, three ORFs (DVU1030, 3298, 3336) were relatively stable throughout exponential, but displayed an increase in expression at the 23 h sampling (Suppl. Fig. 6b). The results suggested that specific putative Usp proteins in *D. vulgaris* might have similar functions during growth transitions and nutrient depletion. Recently, a Usp protein in *E. coli* was shown to specifically complex with GroEL during stationary-phase (8), and both of the putative GroEL and GroES proteins in *D. vulgaris* had increased expression from 23 to 35 h (Suppl. Fig. 7).

Numerous genes predicted to be involved in stress-response were up-expressed as the cells transitioned into stationary-phase. For example, a C-terminal protease (DVU2336) predicted to be located in the periplasm was up-expressed between 23 and 29 h, and remained up-expressed throughout stationary-phase (Suppl. Fig. 8). Two ORFs predicted to encode carbon starvation proteins (DVU0599 and 0598) were up-expressed between 20 and 29 h (Suppl. Fig. 8). The genes were similar to *cst*A in *E. coli*, and CstA is a predicted membrane protein that is involved in peptide utilization during carbon starvation (46).

Carbohydrate-related genes. The increase in carbohydrate levels stopped as the cells transitioned to stationary-phase and actually began to decline (Fig. 2), and the carbohydrate to protein ratio displayed a similar trend. Genes predicted to be involved in carbohydrate metabolism were up-expressed during the phase transition (Fig. 4a). Representative genes that were significantly up-expressed included genes encoding a glycosyl transferase (DVUA0037), lysozyme (DVU1128), a polysaccharide deacetylase (DVUA0043), glycosyl transferase (DVU0351), a sugar dehydratase (DVU0448), an epimerase (DVU2455), glucokinase

(DVU1035), and a sugar facilitator (DVUA0096). Interestingly, three of these genes were located on the megaplasmid of *D. vulgaris*, and preliminary results suggested that carbohydrate profiles in a strain without the megaplasmid differed from wild-type (Fields and Clark, unpublished results). The majority of these genes were up-expressed between 23 and 29 h, and the transcript levels remained elevated into stationary-phase (Suppl. Fig. 9).

The sugar dehydratase (DVU0448) is predicted to be a manno-dehydratase, the enzyme responsible for the first step in the conversion of GDP-mannose to GDP-fucose (28). In *E. coli*, GDP-L-fucose can act as a precursor for surface antigens (e.g., extracellular polysaccharide) (28). In addition, *D. vulgaris* has one putative glucokinase (DVU1035), and the gene was gradually up-expressed until the 43 h sampling. *D. vulgaris* has only 4 genes annotated as probable sugar transporters, and DVUA0096 was the only one to display up-expression during the growth transition into stationary-phase. Few studies have reported the utilization of exogenous sugar in *D. vulgaris*, unlike *Desulfovibrio fructosovorans* which can utilize some sugars (38). The total carbohydrate levels declined more than 2-fold; however, significant amounts of extracellular carbohydrate associated with the cell or in the supernatant were not detected (data not shown). These results suggested a re-routing of internal carbohydrate could occur, but further work is needed to determine the possible role of carbon reserves in *D. vulgaris*.

***In silico* prediction of a stationary-phase promoter sequence**. In order to identify possible transcription factor-binding motifs for stationary-phase responsive genes, the upstream non-coding regions (200 bp) of 136 genes (up-expressed in stationary-phase) were searched for common motifs with the Gibbs Recursive Sampler (30, 52). The algorithms identified 5'-CxGCATGGG-3' with statistical significance ($p < 0.05$). The base frequencies represented by the height of the stacked letters at each position are shown as a sequence logo in Fig. 6. The

presumptive motif is quite different from a recently predicted σ^S consensus promoter element, 5'-TCTATACTTAA-3', for *E. coli* (56). However, it should be noted that *D. vulgaris* does not have a predicted *rpo*S. The function of the possible nucleic acid motif needs to be characterized with experimental approaches.

Discussion

The cell cycle of bacteria has periods of active and slow growth that alternates in relation to nutrient levels and physico-chemical conditions. *In situ* conditions rarely provide excess nutrients for the growth of bacteria, and short periods of growth alternate with prolonged periods of starvation. In the context of *in situ* bioremediation, nutrient scarcity and/or fluxes may be a common obstacle encountered by microorganisms, due to the oligotrophic nature of most groundwater and sediment environments, and require the ability to transition between faster and slower growth. However, cells have mechanisms to survive cycles of nutrient depletion and starvation, as well as other stressful conditions commonly associated with prolonged starvation. The RpoS (σ^S) protein is a major transcriptional activator for cellular responses to stationary-phase growth in many bacteria, and σ^S induces approximately 100 genes in *E. coli* (27). In light of the predicted absence of RpoS in δ-*Proteobacteria*, our results demonstrated that *D. vulgaris* elicited a response to stationary-phase and changes in gene transcription continued throughout.

Bacterial cultures grown in chemostats can provide a semi-steady state for the cultivation of cells at a constant growth rate with one limiting nutrient. While this approach is powerful for physiological studies at a given state, it is not conducive for observations during transitions (e.g., changing levels of carbon and energy sources). Whole-genome transcriptomics have been used to compare regulatory gene mutants (1, 50, 54) and specific stressors (e.g., 14, 18, 31), but only more recently have transcriptomics been used to compare different growth phases or the

respective transitions (5, 7, 37, 47, 49, 55, 56). Most of the previous work has been with *E. coli* or pathogens, and few transcriptomic studies of growth-phase transitions have been reported for 'environmental' microorganisms. *D. vulgaris* is commonly used as a model SRB, and is observed in habitats that are exposed to continuously changing nutritional and environmental conditions. As discussed above, cellular responses to different stressors can overlap with responses to stationary-phase. Because many stressors cause a decrease in growth rate and/or altered nutrient utilization, it is important to elucidate respective responses during the transitions to better understand direct and indirect cellular adaptations.

A summary of the major differentially expressed genes for the respective time points is shown in Fig. 7. The time points T3 and T4 were combined and considered to be late-exponential phase (20 to 23 h) before the transition to stationary-phase. As expected, genes involved with ATP synthase, elongation factors, and ribosomal proteins were down-expressed. Presumptive genes that encode proteins related to carbon starvation and iron acquisition were significantly up-expressed as the cells transitioned. An annotated *kat*A was also up-expressed, as was a putative *psp*A (phage shock protein). PspA is considered to be part of a specialized extracytoplasmic stress response in Gram-negative bacteria (12), and interestingly, most presumptive phage genes were significantly up-expressed at the next time point (29 h) in stationary-phase. Two important inducing conditions for *psp*A are dissipation of the proton motive force (pmf) and mislocalization of secretin proteins that can be used for the extrusion of filamentous phage, and PspA may function to maintain the pmf (12). These results suggested that the depletion of electron donor (carbon and energy source) may have caused a decline in the pmf that affected protein secretion and/or phage cycle.

The cells transitioned into stationary phase at the 29 h sampling (T5), and displayed the largest change in up- or down-expressed genes between successive time points (T4 vs. T5). Between the 35 and 51 h samplings, 197±7 and 105±12 genes were up-expressed and down-expressed, respectively, and the last sampling (67 h) had 250 and 96 genes up- or down-expressed, respectively (Fig. 3). Temporal samples were not analyzed in recent reports with *E. coli* and *Mycobacterium smegatis*, but similar levels of genes (252 and 137 genes, respectively) were described as up-expressed during stationary phase (55, 56). Three time points were analyzed during growth of *Streptococcus pyogenes*, and approximately 125 and 160 genes were up- or down-expressed, respectively (7).

A recent report characterized cellular growth via genomic expression profiles for *Clostridium acetobutylicum* that transitioned from exponential to stationary-phase (5). Similar to our results, ribosomal proteins were down-regulated in response to the transition, but cobalt and iron acquisition systems were up-expressed. In particular, the putative *feo*AB genes were up-expressed as *C. acetobutylicum* transitioned into stationary-phase, and the *D. vulgaris feo* genes displayed the same trend (Table 1). In the case of *D. vulgaris*, Fe^{2+} levels may decline during growth due to the reduced conditions created by H_2S production, and the feo system has been shown to be important for the acquisition of ferrous iron under anaerobic conditions in *E. coli* (25). Recent work in *Legionella pneumophila* showed the importance of Fe^{2+} transport for intracellular replication as a human pathogen (42). The *feo* system may play a similar role of Fe^{2+} acquisition for environmental microorganisms under iron-limited conditions. These results suggested an importance of Fe^{2+} as a nutrient when SRB may be stimulated for bioremediation, however, further work will be needed to determine the role of such systems under *in situ* conditions.

Two of the largest groups of genes with significant changes in expression were ribosomal protein genes and phage-related genes. Some ribosomal protein genes started to display down-expression even at 20 h (half a generation time before cells entered stationary phase), and these results suggested that actively growing cells were down-expressing some ribosomal proteins in response to the decrease in carbon and energy source. Interestingly, the levels of 16 ribosomal proteins drastically declined during the transition, but re-bounded to almost pre-stationary levels by 35 h. This result suggested that even "resting" populations in mid-stationary-phase can have similar levels of ribosomal proteins. Other microorganisms (*Helicobacter, Streptomyces, Clostridium*) have been shown to down-express ribosomal protein genes during the transition to later growth stages (5, 24, 51), but *Chlamydia* do not (36).

Many phage-related genes displayed a dramatic increase in expression during the transition and in stationary-phase for *D. vulgaris*. In a recent study with *S. pyogenes*, phage-related genes were not significantly up-expressed as cells entered stationary phase, and many were actually down-expressed (7). Recently, the lytic cycle of the Mu phage in *E. coli* was shown to be derepressed via the action of ClpXP and Lon proteases on the Rep repressor, and the ClpXP and Lon protease were up-expressed during transition to stationary-phase in *E. coli* (41). The data indicated that phage-related genes were up-expressed in response to a decline in carbon/energy source and/or a decreased growth rate by the host cell. We infer from these results that phage may play an important role in the eco-physiology of *Desulfovibrio* under nutrient-limited conditions. Interestingly, even though phage-related genes were up-expressed, 60% of the biomass still remained after approximately 40 h into stationary phase, and these results suggested that the phage did not cause complete lysis of the entire population.

Lactate and sulfate are two of the major electron donors and acceptors for most *Desulfovibrio* spp., and this inference is supported by the observation of six lactate permeases and three possible sulfate permeases in the annotated *D. vulgaris* genome. The different lactate and sulfate permeases displayed different trends of expression as the cells transitioned between the growth phases. Based upon current annotation, we can deduce that different permeases may be used with respect to changing nutrient levels. For example, DVU3284 might be a low K_m lactate transporter that is up-expressed as lactate levels decline, and DVU0053 and DVU0279 may be low capacity, high affinity and high capacity, low affinity sulfate transporters, respectively. An alternative explanation might be that the sulfate permeases are regulated in a growth-rate dependent manner. A growth-rate dependent mechanism of regulation for the sulfate permeases could be explained by the fact that sulfate can be relatively abundant in most environments compared to electron donors and is the preferred electron acceptor for SRB. A major check point for growth control could be the presence and level of electron donor and not necessarily electron acceptor per se. Further work is needed to determine the individual role(s) for the different transporters, and the environmental conditions under which each is important.

The results indicated that a subset of approximately 110 genes were uniquely up-expressed as the cells transitioned to stationary-phase. The rest of the genes up-expressed during stationary phase were also up-expressed during other stresses (i.e., NO_2, NaCl, pH, and heat) (unpublished results). During the described experiments, the majority of the pH change occurred during exponential growth; however, few genes displayed significant changes in expression through-out exponential growth when compared to the first time point. In addition, the cluster analysis indicated that whole-genome expression profiles changed despite little change in pH during stationary-phase. Particular hypothetical and conserved hypothetical proteins did display

a trend of down-expression that was similar between the stationary-phase response and other stressors, but the exact role of the presumptive gene products is unknown

The subset of up-expressed genes unique to stationary phase was mainly involved with DNA repair, nucleic acid metabolism, amino acid metabolism, and carbohydrate metabolism. Of the unique stationary genes that were up-expressed, 14 (13% of the total) were located on the megaplasmid. In a recent study with *C. acetobutylicum*, megaplasmid (pSOL1) genes had increased expression at the onset of stationary-phase (5). Based on our preliminary results with *D. vulgaris*, a megaplasmid-free strain (△mp) has different growth characteristics in stationary-phase and does not form significant biofilms compared to wild-type cells with the megaplasmid (Clark and Fields, unpublished results).

It should be noted that up to 17 genes (11% of megaplasmid ORFs) on the megaplasmid are predicted to play a role in carbohydrate metabolism, and total carbohydrate levels decreased approximately 2-fold when the cells transitioned to stationary-phase. During the transition, the putative isoamylase and glucan phosphorylase were up-expressed, and a putative glucan synthase and glucan branching enzyme were down-expressed. In addition, the one putative fbp (fructose-1,6-bisphosphatase) for *D. vulgaris* was down-expressed, and this result suggested a carbon flow in glycolysis from glucose towards pyruvate and not gluconeogenesis. Interestingly, the *D. vulgaris* genome has a putative fructose-2,6-biphosphatase (DVU3147), which is a unique characteristic for a bacterium. DVU3147 displayed an upward trend in expression during the transition, and is located immediately upstream from a putative glucotransferase in both *D. vulgaris* and *Desulfovibrio* G20. The fructose-2,6-biphosphatase is thought to be important in the regulation of hepatic carbohydrate metabolism in metazoans (22), but can have other functions in different organisms. The possible role or function in *Desulfovibrio* is not known.

For previously studied bacteria, the transcriptional regulation of gene expression in stationary phase is primarily carried out by σ^S (RpoS). Expression of σ^S is regulated at the level of transcription, translation, and protein degradation (27), and RpoS regulons have been recently delineated via global transcriptomics in *E. coli*, *Pseudomonas fluorescens* Pf-5, and *M. smegatis* (48, 55, 56). It should be noted that *D. vulgaris* does not have an annotated *rpo*S, nor do other δ-*Proteobacteria* including: *Desulfovibrio desulfuricans* G20, *Geobacter sulfurreducens* PCA, *Geobacter metallireducens*, *Bdellovibrio bacteriovorus* HD100, *Desulfotalea psychrophila*, and *Desulfuromonas acetoxidans*. The *D. vulgaris* genome has four putative Rpo factors (*rpo*D, *rpo*H, *rpo*N, and *fli*A), but these genes did not show any significant changes in expression during the growth experiment. These results indicated that *D. vulgaris* cells transitioned to stationary-phase after depletion of a carbon and energy source without a typical σ^S factor and suggested that other factors most likely control gene expression during growth transitions.

When 101 genera with sequenced genomes were checked for the presence of a putative *rpo*S, only 21 had a predicted *rpo*S factor, and these included the following divisions: α-, β-, and γ-*Proteobacteria*, *Bacteroidetes*, *Planctomycetes*, and *Spirochaetes*. These data suggested that RpoS is not a universal transcriptional factor for bacteria, and alternative mechanisms could exist for the regulation of growth transitions in different microorganisms. It is feasible that *D. vulgaris* has a factor that functions as an RpoS, but does not have significant sequence similarity to previously reported sigma factors. Further work is needed to determine the control factor(s) in *D. vulgaris* involved in sensing environmental stimuli, and what cellular systems are required for cells to survive stationary-phase. The elucidation of growth-phase dependent gene expression is essential for a general understanding of growth physiology that is also crucial for data interpretation of stress-responsive genes. In addition, to effectively immobilize heavy metals and

radionuclides via sulfate-reduction, it is important to understand the cellular responses to adverse factors observed at contaminated subsurface environments, such as the changing ratios of electron donors and acceptors. Our results indicated that in addition to expected changes (e.g., energy conversion, protein turnover, translation, transcription, and DNA replication/repair) genes related to phage, carbohydrate flux, outer envelop, and iron homeostasis played a major role in the cellular response to nutrient deprivation under the tested growth conditions.

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Table 1. Representative genes that displayed significant up- or down-expression at the transition to and during stationary-phase growth. Genes with a change in expression of at least 1.50 and with z values ≥ 2.0 are denoted in color (up-expressed, red; down-expressed, blue).

DVU #	Putative Name	T2	T3	T4	T5	T6	T7	T8	T9
2572	feoA	0.16	2.45	3.96	4.05	3.44	3.28	2.78	1.96
2571	feoB	0.18	2.47	4.83	2.47	1.71	1.83	1.54	0.83
1366	lipoprotein	0.07	0.06	0.00	2.50	0.63	0.43	-0.22	-0.73
2861	phage	0.01	0.36	0.21	2.48	3.36	2.68	2.54	2.66
2862	phage	-0.21	0.06	0.03	2.15	2.95	2.74	2.28	2.11
2869	phage	-0.05	0.29	0.30	2.67	3.83	3.68	3.35	2.99
1896	rpsT	-0.62	0.00	-0.72	-4.26	-3.86	-3.75	-4.15	-3.22
1574	rplY	-0.10	-0.55	-1.32	-3.12	-2.11	-2.38	-2.19	-2.36
1575	prsA	-0.29	-0.84	-1.72	-3.42	-1.54	-1.44	-1.70	-1.86
1568	ftn	0.18	0.45	0.47	-2.25	-1.55	-1.90	-1.88	-2.13
0019	ngr	0.39	0.66	0.74	1.90	-0.06	0.37	0.44	0.74
2410	sodB	0.26	0.72	1.10	2.27	1.28	NA	1.33	2.71
1858	csp	0.03	0.51	0.69	5.45	5.76	6.36	6.57	6.66
0938	isoamylase	0.18	0.30	0.62	2.97	2.15	0.84	0.37	0.55
A0037	sugar transferase	-0.12	-0.16	-0.15	3.95	NA	4.68	5.09	3.84
1035	glk	0.35	0.48	0.19	4.59	3.17	4.57	5.49	7.14
A0043	poly. deacetylase	-0.10	NA	NA	5.02	4.20	5.31	6.07	NA
2285	lac.permease	0.06	-0.46	-1.06	-2.34	-2.94	-1.27	-1.43	-2.51
0279	SO$_4$ permease	-0.64	-0.53	-1.02	-2.28	-2.29	-2.49	-1.16	-1.56
0053	SO$_4$ permease	0.32	0.38	0.70	1.49	0.92	0.26	0.15	0.51

Table 2. Primers used for quantitative PCR for the validation of whole-genome microarray results.

Gene	Forward primer (5'-3')	Reverse Primer (5'-3')	Amplicon (bp)
DVU2776	aagtcacttacaagggcaag	gattccttcacgtattccac	100
DVU2571	ccagcttgaagacatggt	gatgtagccgtaacggtagt	100
DVU1311	tcactgccgaagagctta	atgctggaggtgttctcc	103
DVU3108	gtacctcggagacaagatgt	gtcgtccacatcatggaa	105
DVU0014	caagatgcgcaagttctac	tcatacggtaggtgatacgtc	101
DVU1858	gatgacttcgtgtgcagag	tgcacaacgtcatctcac	101
DVU2839	cttagccaagctactagacgac	gctgagttgatgctcaggta	100
DVU2061	agaatctgctggctgaca	gtcgcagtatgccttgat	101

Figure Legends

Figure 1. Protein (■) and carbohydrate (□) levels during growth of *D. vulgaris* cells with lactate (55 mM) and sulfate (35 mM). Arrows denote times at which biomass was removed for RNA extraction. Error bars indicate standard deviation. LE, late-exponential phase; ES, early-stationary phase; MS, mid-stationary phase; LS, late-stationary phase.

Figure 2. Lactate (■), sulfate (•), and pH (◆) levels during growth of *D. vulgaris* with lactate (55 mM) and sulfate (35 mM). Error bars indicate standard deviation. LE, late-exponential phase; ES, early-stationary phase; MS, mid-stationary phase; LS, late-stationary phase.

Figure 3. Total number of ORFs predicted to be up-expressed or down-expressed (Log_2 Ratio $\geq$ 2 with $z \leq 2.0$) when each time point was compared to expression levels at T1. LE, late-exponential phase; ES, early-stationary phase; MS, mid-stationary phase; LS, late-stationary phase.

Figure 4a and b. Up- (**a**) and down-expressed (**b**) genes ($z \geq 2.0$) grouped as COGs (clusters of orthologous genes) with respect to the sampling time point. The following are the listed COGs denoted by color: amino acid transport/metabolism (■), carbohydrate transport/metabolism (■), cell envelope biogenesis (■), cell motility (■), coenzyme metabolism (■), DNA replication/repair (■), energy production (■), inorganic ion transport (■), intracellular trafficking (■), lipid metabolism (■), post-translational modification, (■) signal transduction (■), transcription (■), translation (■). LE, late-exponential phase; ES, early-stationary phase; MS, mid-stationary phase; LS, late-stationary phase.

Figure 5. Hierarchical cluster analysis of sampling time points based upon expression profiles of all predicted ORFs with an expression level of log_2 Ratio $\geq$ 1.5 or $\leq$ -1.5 (**a**). In total, 1181 genes were used for the analysis to compare time points, and a selected portion of the cluster analysis is shown **(b)**.

Figure 6. Sequence logo of a predicted consensus sequence in the 200 bp regions up-stream of up-expressed genes unique to stationary-phase growth in *D. vulgaris*.

Figure 7. General summary of significant up- and down-expressed genes at late-exponential (T3/T4), early stationary (T5), mid-stationary (T6/T7/T8), and late stationary phase (T9) growth).