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Gene and Protein Expression Profiles of *Shewanella oneidensis* during Anaerobic Growth with Different Electron Acceptors

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ABSTRACT

Changes in mRNA and protein expression profiles of *Shewanella oneidenesis* MR-1 during switch from aerobic to fumarate-, Fe(III)-, or nitrate-reducing conditions were examined using DNA microarrays and two-dimensional polyacrylamide gel electrophoresis (2-D PAGE). In response to changes in growth conditions, 121 of the 691 arrayed genes displayed at least a two-fold difference in transcript abundance as determined by microarray analysis. Genes involved in aerobic respiration encoding cytochrome *c* and *d* oxidases and TCA cycle enzymes were repressed under anaerobic conditions. Genes induced during anaerobic respiration included those involved in cofactor biosynthesis and assembly (*moaACE, ccmHF, nosD*, *cysG*), substrate transport (*cysUP, cysTWA, dcuB*), and anaerobic energy metabolism (*dmsAB*, *psrC*, *pshA*, *hyaABC*, *hydA*). Transcription of genes encoding a periplasmic nitrate reductase (*napBHGA*), cytochrome c_{552}, and prismane was elevated 8- to 56-fold in response to the presence of nitrate, while *cymA, ifcA*, and *frdA* were specifically induced three- to eightfold under fumarate-reducing conditions. The mRNA levels for two oxidoreductase-like genes of unknown function and several cell envelope genes involved in multidrug resistance increased two- to fivefold specifically under Fe(III)-reducing conditions. Analysis of protein expression profiles under aerobic and anaerobic conditions revealed 14 protein spots that showed significant differences in abundance on 2-D gels. Protein identification by mass spectrometry indicated that the expression of prismane, dihydrolipoamide succinyltransferase, and alcaligin siderophore biosynthesis protein correlated with the microarray data.

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INTRODUCTION

SHEWANELLA ONEIDENSIS MR-1, formerly known as *S. putrefaciens* MR-1 (Venkateswaran et al., 1999), is a facultatively anaerobic γ-proteobacterium capable of utilizing a wide range of electron acceptors. In recent years, this organism has attracted considerable research interest because of its metabolic versatility and potential for bioremediation of metal contaminants in the environment (Caccavo et al., 1996; Abdelouas et al., 1998; Markwiese et al., 2000; Myers et al., 2000; Taratus et al., 2000; Wielinga et al., 2000). Besides oxygen, *S. oneidensis* can respire various organic and inorganic substrates, including fumarate, nitrate, nitrite, thiosulfate, elemental sulfur, trimethylamine *N*-oxide (TMAO), dimethyl sulfoxide (DMSO), anthraquinone-2,6-disulphonate (AQDS), iron, and manganese (Nealson and Saffarini, 1994; Moser and Nealson, 1996). It has also been shown that *S. oneidensis* is able to reduce uranium [U (IV)] (Lovley, 1991), although there was no indication that metal reduction during this process was coupled to either energy generation or growth.

To utilize these diverse electron acceptors, members of the *Shewanella* genus have developed complex electron transport networks. Early biochemical and genetic studies of *S. oneidensis* have identified various quinone and cytochrome species involved in anaerobic respiration (Obuekwe and Westlake, 1982; Arnold et al., 1986; Akagawa-Matsushita, 1992; Morris et al., 1994). More recently, a variety of cytochromes involved in aerobic and anaerobic respiration have been isolated from different *Shewanella* species (Tsapin et al., 1996; Myers and Myers, 1997, 1998; Dobbin et al., 1999; Gordon et al., 2000). Studies of *S. oneidensis* mutants deficient in metal reduction led to the identification of a 16-kb cluster that encoded genes essential for Fe(III) and Mn(IV) reduction (Beliaev and Saffarini, 1998; Beliaev et al., 2001). Despite the extensive research that has been devoted to anaerobic respiration in *S. oneidensis*, very little is known about the genetic basis and regulatory mechanisms governing the expression of genes involved in electron transport and energy generation.

With the emergence of high-throughput genomic and proteomic technologies, it is now possible to monitor, in parallel, the expression levels of many genes and proteins under different growth conditions (Schena et al., 1996; DeRisi et al., 1997; Ideker et al., 2001; Yoshida et al., 2001). Recently, the 5-Mbp genome of *S. oneidensis* MR-1 was determined by The Institute for Genomic Research (TIGR) under the support of the U.S. Department of Energy, making it feasible to apply microarray technology to the study of energy metabolism in this bacterium. To identify genes and regulatory elements specifically involved in anaerobic respiration, differential mRNA and protein expression profiles of *S. oneidensis* MR-1 were investigated under aerobic, fumarate-, Fe(III)-, and nitrate-reducing conditions using DNA microarrays as well as 2-D PAGE in conjunction with mass spectrometry. Since, at the time of this study, only a preliminary annotation based on an incomplete genome sequence of *S. oneidensis* was available, a subset of 691 genes with putative functions in energy metabolism, transcriptional regulation, biosynthesis, and other cellular functions was selected for representation on microarrays. Both microarray and proteomic analyses revealed substantial differences in expression patterns in response to anaerobic respiratory growth conditions. While the majority of genes and proteins exhibited altered expression levels as a result of transition from aerobic to anaerobic growth, a number of genes displayed preferential induction in the presence of nitrate, fumarate, and Fe(III).

The results of this work represent the first step in characterizing the anaerobic respiratory system of *S. oneidensis* on a genome scale. Given the complexity of the electron transport system of this organism, the sets of data that show coordinate regulation will be the building blocks on which we begin to understand the complex regulation of energy-generating processes and, in fact, will allow us to predict other genes that may behave in similar ways through genomic analysis.

MATERIALS AND METHODS

Strains and growth conditions

S. oneidensis strain MR-1 used in this study was routinely grown in Luria-Bertani (LB) medium (Sambrook et al., 1989) at 25°C. For aerobic growth, 20 mL of LB broth inoculated with 1 mL of overnight culture was placed in a 250-mL flask and incubated on a rotary shaker at a speed of 200 rpm. The cells were

harvested at the exponential phase (OD_{600} = 1). For anaerobic growth, 20 mL of an aerobic exponentially growing *S. oneidensis* culture was used to inoculate 50 mL of anaerobically prepared LB broth supplemented with sodium lactate (20 mM) as an electron donor and the following electron acceptors: 20 mM sodium fumarate, 10 mM ferric citrate, or 5 mM sodium nitrate. Cultures were grown in a Coy anaerobic chamber at 25°C for 8 h and harvested in the exponential growth phase. Each culture was divided into two aliquots, so the same cells were used for both microarray and 2-D PAGE experiments.

Microarray construction

DNA microarrays contained 691 open reading frames (ORFs) of *S. oneidensis* MR-1 putatively involved in energy metabolism, transcriptional regulation, adaptive responses to environmental stress, and substrate transport. A complete list of the *S. oneidensis* MR-1 genes represented on the microarrays is available at www.esd.ornl.gov/facilities/genomics/partial_microarrays.html. Preliminary functional annotations based on sequence homology were assigned to each ORF by TIGR (unpublished data). Polymerase chain reaction (PCR) primers for the amplification of putative genes were designed using the genome sequence information of *S. oneidensis* (TIGR, unpublished data) and the computer program Primer 3 (Whitehead Institute, Cambridge, MA). PCR amplification was considered to be successful if reactions contained a single product of the expected size as determined by agarose gel electrophoresis and ethidium bromide staining. PCR products (100–300 ng/μL) prepared in 50% dimethyl sulfoxide (DMSO; Sigma Chemical Co., St. Louis, MO) were spotted onto organo-modified glass slides (Telechem International, Sunnyvale, CA) with ChipMaker III pins (Telechem International) using a PixSys 5500 robotic printer (Cartesian Technologies, Inc., Irvine, CA). PCR products representing 691 different ORFs were spotted in 4 replicates on a single slide. The following samples were also included on the array: (1) a set of three serial dilutions of *S. oneidensis* MR-1 genomic DNA as positive controls; (2) pUC19 plasmid as a negative control; (3) non–*S. oneidensis* DNA from yeast as an additional negative control; and (4) blank control spots. Arrayed DNA elements were chemically linked to the slides, and the slides were postprocessed according to the manufacturer's instructions (Telechem International).

RNA extraction, labeling, and microarray hybridization

Total cellular RNA from *S. oneidensis* cultures grown under different electron acceptor conditions was isolated using the TRIzol™ Reagent (Gibco BRL, Gaithersburg, MD) according to the manufacturer's instructions. RNA samples were treated with RNase-free DNase I (Ambion, Inc., Austin, TX) to digest residual chromosomal DNA and purified using the Mini RNeasy kit (Qiagen Chatsworth, CA). The concentration and purity of the RNA samples was determined by spectrophotometric measurement at wavelengths of 260 and 280 nm, respectively.

For probe synthesis and labeling, 20 μg of total cellular RNA was incubated at 70°C for 10 min in the presence of 9 μg of random hexamers (Gibco BRL). The labeling reaction was catalyzed by 200 U of Superscript™ II RNase H^- reverse transcriptase (Gibco BRL) in the presence of 500 μM dATP, dGTP, and dTTP; 50 μM dCTP; and 50 μM of the fluorophor Cy3-dCTP or Cy5-dCTP (Perkin Elmer/NEN Life Science Products, Boston, MA). The reverse transcription reaction was allowed to proceed for 2 h at 42°C, followed by RNA hydrolysis in 1 N NaOH at 37°C for 10 min. The labeled cDNA probe was purified immediately using a Qiagen PCR purification column and concentrated in a Savant speedvac centrifuge (Savant Instruments, Inc., Holbrook, NY).

For microarray hybridization, the concentrated labeled probes were resuspended in a 30-μL total volume of hybridization solution that contained 3 × SSC, 0.33% SDS, and 24 μg of unlabeled herring sperm DNA (Gibco BRL). Hybridization was carried out in a waterproof CMT-slide chamber (Corning, Corning, NY) submerged in a 65°C water bath in the dark for 12–15 h. Following hybridization, arrays were washed with 1 × SSC/0.2% SDS and 0.1 × SSC/0.2% SDS for 5 min each and with 0.1 × SSC for 30 sec at ambient temperature prior to scanning.

Quantitative analysis and data processing

Microarrays were scanned using the laser confocal fluorescence microscope of the ScanArray® 5000 Microarray Analysis System (GSI Lumonics, Watertown, MA). Image analysis to determine fluorescence in-

tensity (pixel density) and background intensity, and to identify spots of poor quality was performed using ImaGene™ version 3.0 (Biodiscovery, Inc., Los Angeles, CA). Only those data points with a mean intensity of ≥2 standard deviations above the overall average background for at least one channel were included in the data analysis (Hegde et al., 2000).

Statistical analysis and normalization of the microarray data was performed using the computer program SAS® (SAS Institute, Inc., Cary, NC). Hybridization signal normalization was performed using the geometric mean normalization algorithm (N. Morrison et al., Nature Genetics Microarray Meeting, Scottsdale, Arizona, 1999). Briefly, this included calculating the trimmed geometric mean (TGM) for natural log transformed signal intensities and then calculating $(\ln[X] - \mathrm{TGM[X]}) * (\mathrm{SD}_{\mathrm{TGM}[X]})^{-1}$, where X represents the signal intensity. Values were converted from log space, and expression ratios (Cy5/Cy3) were determined. Natural log-transformed ratios for each experiment were inspected to determine experimental quality and distribution of the ratios. TGM normalized ratios were averaged among replicates, and genes exhibiting at least a twofold change in expression under any of the tested conditions were selected for further analysis (Schena et al., 1996).

Two-dimensional PAGE

Cell pellets of *S. oneidensis* grown aerobically or anaerobically in the presence of fumarate, iron, or nitrate were mixed separately with 5 volumes of a solution containing 9 M urea, 2% 2-mercaptoethanol, 2% pH 8–10 ampholytes (BioRad, Hercules, CA), and 4% Nonidet P40. Cell lysates were centrifuged in a Beckman TL100 ultracentrifuge (435,000 × *g* for 10 min) to sediment all particulates. Protein concentrations were determined using a modified Bradford method (Ramagli et al., 1985), and supernatants were stored at −70°C until analyzed by 2-D gel electrophoresis.

Isoelectric focusing (IEF) gels were cast as previously described (Anderson and Anderson, 1978a) using a 2:1 mixture of pH 5–7 and pH 3–10 ampholytes (BioRad). Aliquots of sample containing 20 μg of total cellular protein were loaded onto each gel. Each sample was subjected to 2-D gel electrophoresis in triplicate to control for gel-to-gel variations and to permit a statistical analysis of the data. Following IEF, the gels were equilibrated in a buffer containing sodium dodecyl sulfate (SDS) as described previously (O'-Farrell, 1975). The second-dimension slab gels were cast using a linear gradient of 10–17% polyacrylamide. The equilibrated tube gels were secured to the slab gels using agarose, and SDS-PAGE was carried out as described (Anderson and Anderson, 1978b). Molecular weight standards (Sigma) included phosphorylase B (97,400), bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (30,000), trypsin inhibitor (21,000), and alpha lactalbumin (14,000). Proteins were fixed in the gels by soaking in a solution containing 50% (vol/vol) ethanol with 0.1% (vol/vol) formaldehyde and 1% (vol/vol) acetic acid for approximately 6 h and subsequently visualized by silver staining (Giometti et al., 1991).

Image acquisition and analysis of two-dimensional gels

The 2-D gel images were digitized using an Eikonix 1412 charge-coupled device scanner interfaced with a VAX 4000-90 workstation. The images were then processed, and parameter lists (spot files) were generated using the Tycho II software developed at Argonne National Laboratory (Anderson et al., 1982). All spot files (two to three two-dimensional gel patterns per biological sample) were compared so that each matched spot in the patterns was numbered. Statistical analysis of the relative abundance of each matched protein spot across the data set was accomplished by using a two-tailed *t* test as described previously (Giometti and Taylor, 1991). Only those proteins showing quantitative differences with at least a probability (p) of less than 0.05 were considered to differ significantly in abundance.

Protein identification by mass spectrometry

Aliquots of MR-1 samples containing 150–200 μg of protein were separated by 2-D PAGE and the proteins were detected by using Coomassie Blue R250. Proteins to be identified were cut from one to five replicate gels (number of spots required varied with the abundance of individual proteins), reduced at room temperature with tris(2-carboxyethyl) phosphine (Pierce, Rockport, IL), alkylated with iodoacetamide (Sigma), and digested *in situ* with modified trypsin (Promega Corp., Madison, WI). The resulting peptides

were eluted from the gel with 25 mM ammonium bicarbonate and 5% formic acid in 50% acetonitrile and then analyzed by micro liquid chromatography-electrospray ionization tandem mass spectrometry (μ-LC-ESI-MS/MS; Shevchenko et al., 1996).

For μ-LC-ESI-MS/MS, samples were loaded onto a 365 $\times$ 100 μm fused silica capillary (FSC) column (Gatlin et al., 1998) packed with 5 μm of Zorbax XDB-C18 packing material (Agilent Technologies, Palo Alto, CA) at a length of 7–8 cm. The tryptic peptides were separated with a 30-min linear gradient of 0–60% solvent B (80% acetonitrile/0.02% heptafluorobutyric acid), and then entered a LCQ ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA). Tandem mass spectra were automatically collected under computer control during the 30-min LC-MS runs. MS/MS spectra were then directly subjected to SEQUEST (Eng et al., 1994) database searches without the need for manual interpretation. SEQUEST identified proteins in a spot by correlating experimental MS/MS spectra to protein sequences predicted by the *S. oneidensis* MR-1 ORF database (TIGR, unpublished results).

RESULTS AND DISCUSSION

Reproducibility of microarray hybridization

To explore gene expression in *S. oneidensis* under anaerobic growth conditions, DNA microarrays representing approximately 16% of the total protein-coding capacity of the MR-1 genome were constructed. The reproducibility of microarray hybridization was assessed according to a previously described method (Khodursky et al., 2000). Two anaerobic cultures inoculated with exponentially growing aerobic *S. oneidensis* culture were grown in parallel under fumarate-reducing conditions for 8 h. The expression ratios for each gene were determined in independent hybridization experiments and plotted against each other to determine the correlation between slide (technical) and mRNA (biological) replicates (Fig. 1). We found a high degree of reproducibility between technical and biological replicate experiments with Pearson correlation coefficients (r) of 0.94 and 0.88 ($p < 0.001$), respectively (Fig. 1). The variation between technical replicates was lower than the variation between biological replicates as demonstrated by the tighter grouping of points and the higher correlation coefficient. This is not surprising since the biological replicates include additional sources of variability such as slight differences in growth conditions and RNA extraction that do not exist in the slide replicates. Our data suggest that microarray hybridization yields reproducible results; however, biological replicates are needed for alleviating inherent higher variation associated with microarray experiments.

In this study, two biological replicates were used in the gene expression analysis for all growth conditions. For each mRNA replicate, a reference aerobic culture of exponentially growing *S. oneidensis* MR-1 was used to inoculate the anaerobic cultures supplemented with fumarate, ferric iron, or nitrate. Two independent hybridization experiments were carried out using the same biological mRNA sample, with each slide containing four replicates of the array. After image analysis and signal normalization, the filtered data set for each condition contained a total of eight to 16 data points per gene.

Expression of genes involved in electron transfer

Microarray expression profiling of *S. oneidensis* grown with different electron acceptors revealed a total of 121 genes that exhibited at least a twofold change in mRNA abundance during the transition from aerobic to anaerobic metabolism (Table 1). Out of 121, forty genes were involved in electron transport. This group consisted mostly of genes whose roles in energy metabolism are well documented in other organisms (Baker et al., 1998; Smith et al., 2000; Unden and Bongaerts, 1997). Based on microarray expression patterns, the electron transport genes can be divided into three categories. The first category consisted of genes associated with aerobic respiration including electron transfer flavoproteins as well as a cytochrome *d*-oxidase and two cytochrome *c*-oxidases (Table 1). The transcription of genes in this subgroup was repressed two- to eightfold under the anaerobic conditions tested. The second category of genes, containing anaerobic oxidoreductases, *c*-type cytochromes, and dehydrogenases, was induced under all anaerobic conditions tested, although the highest expression levels were generally observed when fumarate was used as

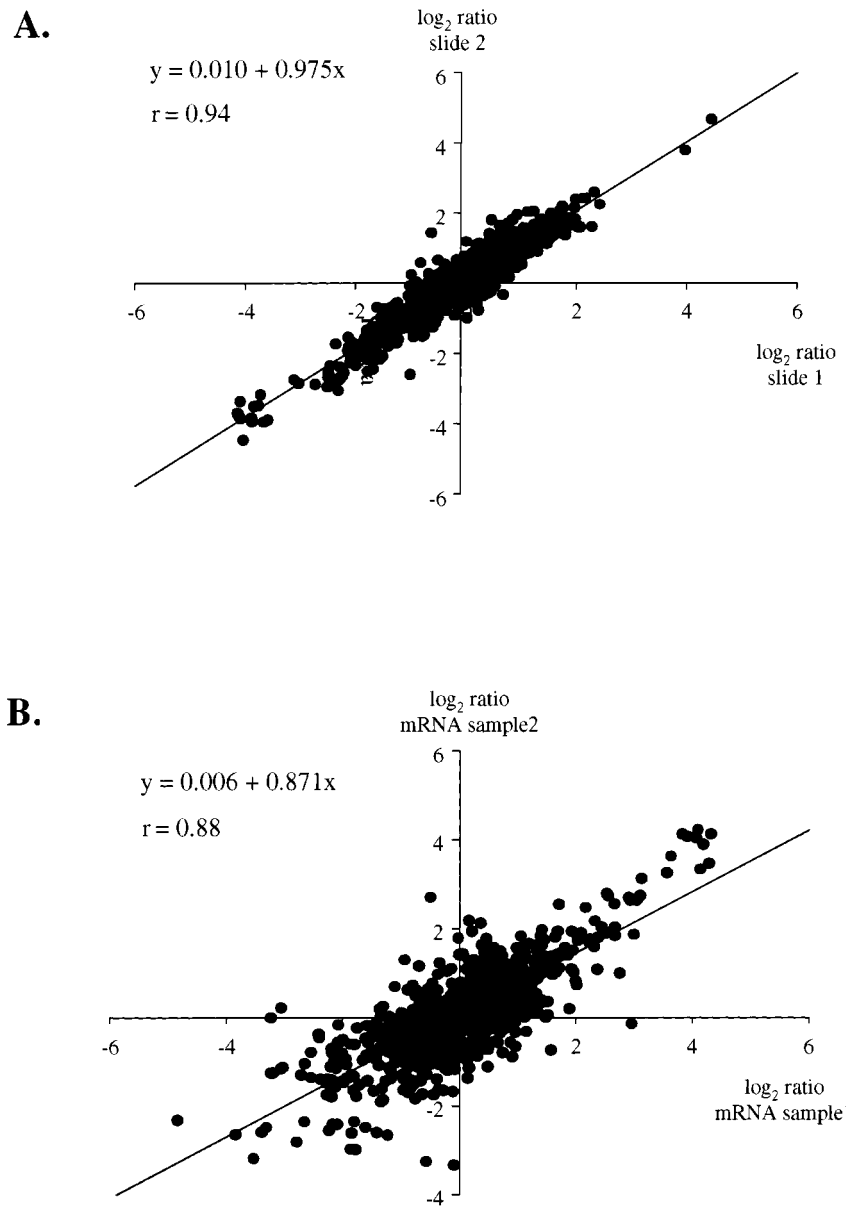


FIG. 1. Scatter plot of fluorescence ratios (anaerobic$_*$aerobic^{-1}) obtained from technical replicates (different slides, same mRNA sample) (**A**) and biological (different mRNA samples) replicates (**B**). In each experiment, the fluorescent ratios measured for each gene in the two independent hybridizations were compared to each other, using the correlation between two complete data sets (the log base 2 ratio for each gene) as a measure of reproducibility. The linear regression models and Pearson correlation coefficients (r) are shown for each experiment.

the electron acceptor. Most of the genes in this group, such as those encoding quinone-reactive Ni/Fe-containing hydrogenase (ORFs 1142 and 1143) and periplasmic [Fe] hydrogenase (ORFs 4138–4142), proteins involved in metal reduction (ORFs 3454 and 3455), and polysulfide/thiosulfate reductase (ORFs 1280 and 1282), showed a moderate increase in mRNA abundance ranging from two- to fivefold (Table 1). The highest induction ratios were exhibited by the dimethyl sulfoxide reductase cluster (ORFs 3454–3458), selenocysteine-containing formate dehydrogenase and cytochrome c_{551} peroxidase gene, which increased 8- to 16-fold under fumarate-reducing conditions and 3- to 10-fold under iron- and nitrate-reducing conditions (Table 1).

Table 1. Changes in Expression Levels of *S. oneidensis* MR-1 Genes Involved in Electron Transport during Transition from Aerobic to Anaerobic Conditions[a]

ORF number/putative function[b]	*Fumarate*	*Fe(III)*	*Nitrate*
(1) Electron transport genes			
Genes repressed under anaerobic conditions:			
343—Electron transfer flavoprotein, α-subunit	−2.79 (±1.45)	−8.01 (±4.24)	−2.03 (±0.24)
344—Electron transfer flavoprotein, β-subunit	−3.50 (±1.26)	−4.96 (±2.22)	−1.92 (±0.30)
487—Cytochrome *d* ubiquinol-oxidase, subunit II	−2.34 (±0.33)	−3.77 (±0.53)	−2.10 (±1.26)
488—Cytochrome *d* ubiquinol-oxidase, subunit I	−2.70 (±1.12)	−5.49 (±0.55)	−3.03 (±1.50)
1848—Cytochrome *c*-oxidase, subunit II	−4.44 (±1.58)	−3.55 (±0.24)	−4.11 (±1.89)
1850—Cytochrome *c*-oxidase, subunit I	−5.46 (±0.82)	−3.42 (±0.48)	−3.88 (±1.73)
1852—Cytochrome *c*-oxidase, subunit III	−6.94 (±0.12)	−5.13 (±0.59)	−7.69 (±1.54)
4403—*cbb*3-type cytochrome *c*-oxidase, *ccoP*	−3.07 (±0.78)	−3.75 (±0.83)	−2.46 (±1.93)
4404—Cytochrome *c*-oxidase, *ccoQ*	−4.66 (±2.17)	−4.71 (±1.17)	−4.16 (±3.66)
Genes induced under anaerobic conditions:			
236—Flavocytochrome c, flavin subunit	−1.16 (±0.51)[c]	1.44 (±0.34)	3.18 (±0.83)
1142—Periplasmic [Fe]-hydrogenase large subunit, *hydA*	2.13 (±0.76)	2.34 (±0.18)	1.83 (±0.49)
1143—Hydrogenase I	5.23 (±1.62)	5.64 (±1.75)	3.61 (±0.64)
1203—Cytochrome c_{552} precursor	1.56 (±0.65)	1.95 (±0.35)	13.94 (±5.85)
1280—Polysulfide reductase chain C, *psrC*	1.23 (±0.19)	4.84 (±2.10)	3.79 (±1.04)
1282—Thiosulfate reductase precursor, *pshA*	2.92 (±1.62)	4.76 (±1.40)	4.37 (±1.61)
1752—Formate dehydrogenase, selenocysteine	10.98 (±5.88)	5.32 (±0.99)	3.75 (±0.38)
1754—Formate dehydrogenase, cyt. b_{556}, *fdnI*	13.88 (±5.27)	6.60 (±1.17)	2.63 (±1.50)
1835—Tetra-heme cytochrome *c*, *cymA*	7.57 (±4.10)	1.87 (±0.06)	2.32 (±0.85)
1863—Fumarate reductase, flavocytochrome c_3	2.81 (±1.87)	1.77 (±0.22)	1.90 (±0.69)
2848—Periplasmic nitrate reductase small subunit, *napB*	2.67 (±0.23)	2.48 (±0.23)	18.25 (±6.40)
2849—Ferredoxin-type protein, *napH*	1.28 (±0.10)	1.30 (±0.75)[c]	7.68 (±1.99)
2850—Ferredoxin-type protein, *napG*	1.86 (±0.56)	2.45 (±0.36)	13.63 (±2.95)
2851—Periplasmic nitrate reductase precursor, *napA*	1.82 (±1.03)	3.19 (±1.04)	24.29 (±9.15)
2987—Fumarate reductase, flavoprotein subunit, *frdA*	4.77 (±1.47)	1.41 (±0.19)	1.56 (±0.64)
3280—Probable oxidoreductase, *ordL*	1.26 (±0.03)	2.40 (±0.18)	−1.16 (±0.01)
3290—Conserved hypothetical protein, *ordL*-like	1.43 (±0.63)	2.28 (±0.25)	−1.03 (±0.14)[c]
3388—Prismane	1.78 (±0.06)	2.00 (±2.21)[c]	55.59 (±0.74)
3449—Flavocytochrome *c*, *ifcA*	2.38 (±0.10)	1.32 (±0.16)	1.37 (±0.05)
3454—Deca-heme cytochrome *c*, *mtrA*-like	8.73 (±0.03)	3.54 (±0.25)	4.34 (±1.95)
3455—Outer membrane protein precursor, *mtrB*-like	15.86 (±2.28)	7.07 (±0.58)	9.52 (±1.65)
3457—Anaerobic DMSO reductase, α-subunit, *dmsA*	18.18 (±0.77)	8.35 (±1.03)	10.08 (±4.06)
3458—Anaerobic DMSO reductase, β-subunit, *dmsB*	16.66 (±0.25)	8.92 (±0.74)	6.52 (±0.57)
3805—Outer membrane protein precursor, *mtrB*	4.74 (±0.95)	2.05 (±0.23)	1.42 (±0.20)
3806—Deca-heme cytochrome *c*, *mtrA*	5.05 (±0.78)	2.74 (±0.10)	1.84 (±0.49)
3808—Deca-heme cytochrome *c*, *omcA*	4.56 (±2.13)	2.15 (±0.30)	2.25 (±1.17)
4138—Quinone-reactive Ni/Fe-hydrogenase, small subunit	2.82 (±2.40)	2.91 (±0.68)	2.63 (±0.64)
4141—Ni/Fe- hydrogenase, *hyaB*	4.71 (±2.73)	2.00 (±0.32)	2.17 (±1.03)
4142—Quinone-reactive Ni/Fe-hydrogenase, cyt.*b*, *hyaC*	3.30 (±0.88)	2.13 (±0.97)	2.29 (±0.65)
4215—Cytochrome c_{551} peroxidase, *yhjA*	11.51 (±1.38)	3.70 (±0.27)	4.44 (±1.46)
(2) Intermediary carbon metabolism genes			
TCA cycle:			
2336—Citrate synthase	−3.96 (±0.99)	−4.50 (±1.06)	−3.26 (±2.76)
3957—Succinate DH putative iron sulphur subunit, *sdhB*	−2.96 (±0.77)	−1.99 (±0.16)	−2.59 (±1.18)
3958—2-Oxoglutarate dehydrogenase e1 component, *sucA*	−3.61 (±1.84)	−1.85 (±0.25)	−2.94 (±1.20)
3959—Dihydrolipoamide succinyltransferse, *sucB*	−3.31 (±1.61)	−2.38 (±0.21)	−2.61 (±1.18)
3960—Succinyl-Coa synthetase, *sucC*	−3.55 (±2.88)	−4.28 (±0.58)	−2.87 (±1.81)
4384—Dihydrolipoamide acetyltransferase, *aceF*	−3.08 (±0.81)	−2.08 (±0.32)	−2.62 (±0.72)
4813—Acetyl-CoA synthetase, *acs-I*	−4.36 (±0.15)	−5.74 (±1.47)	−3.27 (±0.46)

(*Continued*)

Table 1. (Cont'd) Changes in Expression Levels of *S. oneidensis* MR-1 Genes Involved in Electron Transport during Transition from Aerobic to Anaerobic Conditions[a]

ORF number/putative function[b]	*Fumarate*	*Fe(III)*	*Nitrate*
Glyoxilate bypass:			
1951—Malate synthase-related	−2.22 (±0.80)	−1.94 (±0.16)	−1.64 (±0.07)
3511—Malate synthase, *aceB*	−2.33 (±0.95)	−3.42 (±1.09)	−2.56 (±1.28)
3512—Isocitrate lyase, *aceA*	−7.17 (±1.67)	−7.72 (±3.01)	−4.14 (±1.04)
Other intermediary pathways:			
76—Phosphotransacetylase	4.42 (±0.80)	1.25 (±0.17)	1.34 (±0.33)
352—Putative acetyl transferase	−3.32 (±0.24)	−5.60 (±2.62)	−2.42 (±2.27)
1073—Malate oxidoreductase	−2.25 (±0.53)	−2.14 (±0.24)	−1.45 (±0.04)
1724—Aldehyde dehydrogenase, *aldA2*	−2.28 (±0.05)	−4.34 (±1.53)	−1.98 (±0.56)
2287—Phosphoglycolate phosphatase, *gph*	2.23 (±0.19)	2.08 (±0.28)	2.25 (±0.22)
3566—NAD^+-isocitrate dehydrogenase, α subunit	−1.95 (±0.47)	−2.22 (±0.32)	−2.27 (±0.41)
3779—Phosphomannomutase, putative	−2.22 (±0.48)	−3.27 (±0.40)	−2.23 (±0.57)
3789—Streptogramin A acetyl transferase, *cat*	−3.07 (±0.27)	−2.59 (±0.28)	−2.59 (±0.38)
3920—β-ketoadipate:succinyl-CoA transferase	−4.77 (±2.69)	−7.18 (±2.65)	−5.16 (±1.98)
3931—Propionyl-CoA synthetase, *prpE*	−3.35 (±1.22)	−3.41 (±0.80)	−3.33 (±0.63)
(3) Regulatory genes			
363—Sensor histidine kinase	−2.96 (±0.80)	−4.66 (±0.19)	−2.47 (±1.94)
433—Two-component response regulator, *flaM*	−2.55 (±1.16)	−3.05 (±0.34)	−2.35 (±0.20)
434—Sensor kinase, PAS domain-containing	−1.92 (±0.38)	−2.51 (±0.37)	−2.04 (±0.25)
795—Transcriptional regulator, *ompR*	−2.71 (±0.01)	−2.97 (±0.47)	−1.65 (±0.30)
1204—Nitrate/nitrite sensor protein, *narQ*	2.28 (±0.25)	1.80 (±0.25)	2.25 (±0.22)
1382—Sensor protein, tetrathionate respiration, *ttrS*	3.35 (±0.73)	4.63 (±0.32)	1.41 (±0.22)
1476—Transcriptional regulator, *tetR* family	1.92 (±0.46)	2.73 (±0.82)	1.75 (±0.31)
1845—LexA repressor	1.01 (±0.36)[c]	2.97 (±0.30)	1.15 (±0.03)
2099—Histidine utilization repressor, *hutC*	2.13 (±0.70)	1.94 (±0.36)	2.20 (±0.30)
2141—Bacterial extracellular solute-binding protein	2.47 (±0.69)	2.75 (±0.25)	2.19 (±0.09)
2653—Positive regulator of late transcription	−1.26 (±0.77)[c]	4.29 (±1.56)	1.56 (±1.22)[c]
3281—Probable transcriptional regulator	2.70 (±0.18)	3.33 (±0.83)	1.32 (±0.18)
4015—Transcriptional activator, putative	−3.55 (±1.95)	−3.40 (±0.32)	−2.56 (±0.84)
4016—RNA polymerase, σ 70, ECF family	−1.87 (±0.41)	−2.06 (±0.27)	−2.04 (±0.08)
4258—Unknown function, PAS domain-containing	−3.48 (±1.60)	−2.44 (±0.50)	−2.57 (±1.12)
4503—Sensor histidine kinase	−2.25 (±0.07)	−2.09 (±0.38)	−1.91 (±0.48)
4794—Probable *csgAB* operon transcriptional regulator	−2.26 (±0.79)	−3.56 (±0.67)	−2.34 (±0.93)
(4) Substrate transport and binding genes			
207—AlcC protein	−8.51 (±8.44)	−23.51 (±7.60)	−5.39 (±5.65)
208—Ferric alcaligin siderophore receptor, *fhuE*	−7.12 (±2.82)	−9.33 (±0.58)	−12.57 (±11.01)
209—Ferric hydroxamate transport protein, *fhuF*	−10.17 (±1.70)	−12.91 (±4.28)	−9.69 (±2.55)
801—Thiosulfate ABC-transporter, *cysP*	2.52 (±1.82)	5.80 (±0.43)	4.13 (±4.06)
802—Sulfate ABC-transporter, *cysU*	1.54 (±1.31)[c]	2.43 (±0.54)	2.51 (±1.85)
870—Heme-hemopexin utilization protein C, *hutA*	−7.64 (±2.93)	−18.58 (±6.86)	−5.41 (±4.15)
871—TonB protein, putative	−5.34 (±1.14)	−8.98 (±0.33)	−4.33 (±2.19)
1655—Anaer. C_4-dicarboxylate membrane transporter, *dcuB*	4.99 (±2.20)	3.94 (±0.74)	2.31 (±0.40)
1755—Vulnibactin outer membrane receptor, putative	−3.79 (±3.14)	−5.31 (±2.37)	−1.72 (±1.01)
1761—Iron-regulated OM virulence protein, *irgA*	−3.93 (±1.65)	−8.84 (±0.95)	−1.74 (±1.82)[c]
1805—TonB2, putative	−3.13 (±2.75)	−8.27 (±1.03)	−1.58 (±1.65)[c]
1897—Sulfate transport protein, CysT	1.08 (±0.34)[c]	3.80 (±0.10)	1.54 (±0.37)
1898—Sulfate transport protein, CysW	1.60 (±0.72)[c]	4.16 (±0.74)	1.45 (±0.68)
1899—Sulfate transport protein, CysA	1.84 (±1.12)	2.95 (±0.13)	2.07 (±2.12)[c]
1988—Ferrichrome-iron receptor	−1.49 (±0.22)	−5.23 (±0.51)	−1.53 (±0.04)

(*Continued*)

TABLE 1. (CONT'D) CHANGES IN EXPRESSION LEVELS OF *S. ONEIDENSIS* MR-1 GENES INVOLVED IN ELECTRON TRANSPORT DURING TRANSITION FROM AEROBIC TO ANAEROBIC CONDITIONS[a]

ORF number/putative function[b]	*Fumarate*	*Fe(III)*	*Nitrate*
2304—Outer membrane porin, *ompF*-like	7.00 (±4.62)	5.37 (±0.35)	9.11 (±0.03)
2482—Copper-binding periplasmic protein, *nosD*	2.26 (±0.67)	3.14 (±0.91)	3.04 (±1.28)
3509—Ferrichrome-iron receptor, putative	−1.03 (±0.16)[c]	−3.12 (±0.88)	1.15 (±1.06)[c]
3607—Ton-dependent heme receptor A, putative	−1.27 (±0.78)	−4.17 (±0.69)	−3.50 (±0.74)
3704—Outer membrane protein W precursor	−5.21 (±0.81)	−11.87 (±1.96)	−4.36 (±2.65)
3850—Outer membrane porin *ompF*-like	3.82 (±0.01)	4.34 (±0.24)	3.87 (±0.37)
3857—TonB2	−1.21 (±0.01)	−2.27 (±0.32)	−1.61 (±0.12)
(5) Genes involved in drug resistance, biosynthesis, and other cellular processes			
Cell envelope and outer membrane:			
289—Acriflavin resistance protein, *acrA/acrE* family	−1.74 (±0.16)	−2.50 (±0.25)	−2.44 (±0.12)
1448—Penicillin-binding protein 3, *ftsI*	1.55 (±0.36)	2.89 (±0.63)	1.48 (±0.22)
2520—Probable multidrug resistance protein, *vceB*	1.55 (±0.44)	5.79 (±0.56)	1.71 (±0.41)
2631—Penicillin-binding protein 1B, *mrcB*	1.44 (±0.29)	2.83 (±0.44)	1.49 (±0.33)
3181—D-alanyl-D-alanyl carboxypeptidase, *dacA-1*	1.10 (±0.21)[c]	2.15 (±0.20)	1.66 (±0.40)
3186—Penicillin-binding protein 2, *mrdA*	1.46 (±0.59)	2.55 (±0.31)	1.80 (±0.54)
4429—Multidrug resistance protein D, *emrD-2*	1.76 (±0.53)	2.83 (±0.55)	1.46 (±0.38)
Cofactor biosynthesis and assembly:			
930—Siroheme synthase, *cysG*	2.53 (±0.63)	3.14 (±1.32)	5.41 (±4.76)
1053—Glutamyl-tRNA reductase, *hemA*	2.46 (±0.04)	2.44 (±0.21)	1.99 (±0.46)
1692—Molybdenum cofactor biosynthesis protein E, *moaE*	2.50 (±1.89)	1.73 (±0.21)	1.48 (±0.27)
1694—Molybdenum cofactor biosynthesis protein C, *moaC*	1.97 (±0.13)	1.58 (±0.19)	1.65 (±0.40)
1695—Molybdenum cofactor biosynthesis protein A, *moaA*	2.03 (±0.35)	1.92 (±0.23)	1.45 (±0.38)
2263—Cytochrome *c* biogenesis, *ccmH*	2.21 (±0.29)	3.13 (±0.22)	2.60 (±0.66)
2264—Cytochrome *c* biogenesis, *ccmF*	2.44 (±0.01)	2.31 (±0.43)	2.61 (±1.10)
2428—Uroporphyrinogen decarboxylase, *hemE*	2.97 (±1.21)	2.19 (±0.10)	3.17 (±1.09)
DNA metabolism:			
2601—DNA mismatch repair protein, *mutL*	1.37 (±0.39)	3.37 (±0.14)	1.23 (±0.18)
Deoxyribonucleotide metabolism:			
4910—Anaerobic rNTP reductase activating protein, *nrdG*	2.60 (±0.38)	4.89 (±0.69)	2.27 (±0.69)
4911—Anaerobic rNTP reductase, *nrdD*	6.27 (±2.52)	9.07 (±0.58)	7.82 (±2.33)
Fatty acid metabolism:			
2079—Long chain fatty acid coA ligase, putative	1.62 (±0.50)	3.06 (±0.09)	1.77 (±0.47)
Protein metabolism/degradation:			
1164—Serine protease, *htrA*-like	−1.75 (±1.18)	2.95 (±0.30)	−1.68 (±0.93)
1165—Protease, *degS*	−1.11 (±0.31)[c]	4.48 (±0.35)	1.28 (±0.23)
Amino acid biosynthesis:			
61—Cysteine synthase A, *cysK*	2.32 (±1.75)	3.36 (±0.38)	3.44 (±0.36)
Detoxification:			
550—Glutathione peroxidase, probable	−1.73 (±0.46)	−2.77 (±1.42)	−2.96 (±1.47)

[a]Relative expression is presented as the ratio of the dye intensity of the anaerobically grown cultures to that of the aerobic reference. Positive values indicate induction fold under anaerobic conditions; negative values indicate the repression fold under anaerobic conditions (inverse negative value of the relative expression ratio). The data are mean values derived from eight to 12 total replicates. The standard deviation for each mean expression ratio is provided in parenthesis.

[b]Sequence annotation was provided as a courtesy of TIGR (unpublished results). The ORF numbers provided here are for the purpose of tracking gene locations and annotation. The ORF numbers will change once the complete version of the *S. oneidensis* MR-1 genome sequence annotation is released.

[c]The absolute value of the mean ratio is not significantly different from 1 at $p = 0.05$ based on a one-tailed t test.

The third category of genes included those whose expression levels were preferentially induced in response to fumarate, iron, or nitrate. During nitrate respiration, six genes displayed substantial induction ratios from 8- to 56-fold (Table 1). These genes included prismane (ORF 3388), cytochrome c_{552} component of the nitrite reductase (ORF1203), and a periplasmic nitrate reductase cluster, *napBGHA* (ORFs 2848–2851). While roles of NrfA and NapBGHA in anaerobic respiration have been well defined in several organisms (Darwin et al., 1993; Hussain et al., 1994; Potter and Cole, 1999), the physiological function of prismane has yet to be determined. Prismane, which is a hybrid iron-sulfur cluster protein (Pierik et al., 1992; Stokkermans et al., 1992), was detected under nitrate/nitrite-reducing conditions in other facultative anaerobes, such as *E. coli* and *Morganella*, suggesting an involvement in nitrate and/or nitrite respiration (van den Berg et al., 2000).

Four genes were induced when fumarate was used as the electron acceptor. The transcription of *cymA* (ORF 1835 in Table 1), which encodes a periplasmic tetraheme *c* cytochrome involved in anaerobic respiration in *S. oneidensis* (Myers and Myers, 1997), was increased eightfold under fumarate-reducing conditions. Lower-fold induction ratios (e.g., two- to fivefold) were observed for three flavocytochrome genes (ORFs 1863, 2987, and 3449; Table 1). One of these genes (ORF 3449), a putative flavocytochrome c_3, shares 41% sequence identity at the amino acid level with the Ifc_3 protein isolated from *S. frigidimarina* (Dobbin et al., 1999). In contrast to Ifc_3, which is expressed specifically under iron-reducing conditions, the flavocytochrome c_3 from *S. oneidensis* displayed over twofold induction in response to anaerobic growth with fumarate, while its transcription remained unaffected in the presence of nitrate or iron.

Under iron-reducing conditions, a marginal increase of approximately twofold was observed for genes encoding a conserved hypothetical protein (ORF 3290) and a putative oxidoreductase (ORF 3280) of unknown function (Table 1). Preliminary sequence analysis of the conserved hypothetical protein revealed 76% identity to another hypothetical protein (GenBank accession number PA2776) from *Pseudomonas aeruginosa* PAO1 and 67% identity to a putative OrdL oxidoreductase (GenBank accession number U38543) from *E. coli*. The deduced amino acid sequence of the putative oxidoreductase gene was 59% identical and 74% similar to the *ordL* gene product (GenBank accession number PA5309) from *P. aeruginosa* PAO1. In addition, both proteins from *S. oneidensis* contained a putative *N*-terminal FAD-binding domain (Vallon, 2000), and their sequences shared 40% identity at the amino acid level, which may be indicative of their functional relatedness. Mutagenesis studies will be conducted to elucidate the potential involvement of these genes in iron reduction.

Transcription of genes involved in intermediary carbon metabolism

S. oneidensis MR-1 can use glucose, lactate, pyruvate, propionate, acetate, ethanol, and a number of carboxylic and amino acids when grown aerobically. Previous studies of carbohydrate metabolism in this organism suggested that glucose utilization proceeds via the Entner-Doudoroff pathway with formation of acetyl-CoA, which is subsequently oxidized through the tricarboxylic acid (TCA) cycle (Scott and Nealson, 1994).

To determine whether the expression of *S. oneidensis* genes associated with carbon utilization is affected by anaerobiosis, we measured the transcription levels of 20 genes putatively involved in intermediary carbon metabolism. Of this subset, 18 genes reproducibly displayed two- to eightfold decreases in mRNA abundance under fumarate-, iron-, and nitrate-reducing conditions (Table 1). Based on the preliminary sequence annotation, seven of these genes grouped within the putative TCA cycle category; three genes fell within the glyoxylate bypass category, and nine genes encoded proteins implicated in other intermediary carbon pathways. The lowest transcription levels under anaerobic conditions were exhibited by homologs of citrate synthase (ORF 2336), succinyl-CoA synthetase (ORF 3960), acetyl-CoA synthetase (ORF 4813), isocitrate lyase (ORF 3512), and β-ketoadipate:succinyl-CoA transferase (ORF 3920) genes. Our results agreed with previous biochemical studies suggesting that the activities of key TCA cycle enzymes are reduced under anaerobic conditions (Scott and Nealson, 1994). We compared the microarray data to the citrate synthase and 2-oxoglutarate dehydrogenase activities measured in both aerobic and nitrate-reducing *S. oneidensis* cultures (Scott and Nealson, 1994) and found a good correlation between mRNA abundance ratios and enzyme activity levels (data not shown).

Under anaerobic conditions, formate instead of acetate is the key intermediate compound (Scott and Nealson, 1994). Substrates such as lactate and pyruvate, which can be converted to formate, are assimilated at

the level of formaldehyde via a modification of the serine pathway found in type II methanotrophs (Hanson and Hanson, 1996), while acetate is excreted into the medium. Two genes induced by anaerobiosis encoded a putative phosphate acetyltransferase and phosphoglycolate phosphatase. The mRNA abundance of phosphate acetyltransferase, an enzyme converting acetyl-CoA into acetate, displayed a fourfold increase specifically under fumarate-reducing conditions (Table 1). The transcription levels of the putative phosphoglycolate phosphatase, responsible for the interconversion of 2-phosphoglycolate to glycolate, were increased twofold under the anaerobic conditions examined. Although the representation of carbon metabolism genes was limited in our study, the expression patterns of those genes present on the partial genome microarray were in accordance with the pathway previously proposed for *S. oneidensis* (Scott and Nealson, 1994). A comprehensive analysis of intermediary carbon metabolism will be possible upon completion of the *S. oneidensis* genome sequencing project.

Expression profiles of regulatory genes

Among the regulatory genes displaying differences in expression under anaerobic growth conditions, more than 30% encoded two-component signal-transducing sensors and response regulators (Table 1). Genes repressed under anaerobic conditions included a number of two-component sensors and DNA-binding proteins. Under anaerobic growth conditions, decreases of two- to threefold in transcription were also observed for a histidine utilization repressor (ORF 2099), a sigma 70 RNA polymerase operon (ORF 4503), and a histidine sensor kinase (ORF 4503). Two other sensor kinase genes (ORFs 434 and 4258) exhibiting a two- to threefold decrease in mRNA abundance under anaerobic conditions contained putative PAS domains, which are important sequence motifs of signal transduction systems that sense changes in oxygen concentration, redox potential, light and the overall energy level of the cell (Bespalov et al., 1996; Taylor and Zhulin. 1999). Of these genes, ORF 434 appears to form an operon with a putative sigma 54-dependent response regulator sharing 62% identity with FlaM (ORF 433) of *Vibrio parahaemolyticus*, which is located in the middle of the 50-kb flagella gene cluster of *S. oneidensis*.

Among regulatory genes induced by anaerobiosis, we identified two ORFs encoding signal-transducing proteins involved in substrate-mediated responses. A putative nitrate/nitrite sensor (ORF 1204), which shares 35% sequence identity with the NarQ protein from *E. coli* (Rabin and Stewart. 1992), exhibited a twofold increase under anaerobic conditions. The other protein (ORF 1382), a putative tetrathionate sensor TtrS, was induced three- and fourfold under fumarate- and iron-reducing conditions, respectively (Table 1). Several regulatory genes encoding a protein similar to LexA (ORF 1845; Little and Harper, 1979), a TetR-family transcriptional regulator (ORF 1476; Beck et al., 1982), and a positive regulator of late transcription (ORF 2653), exhibited three- to fourfold increases in mRNA abundance specifically under iron-reducing conditions. We cannot explain, however, why increases in transcript levels were observed for these three genes, and further research is required to determine whether these regulators are actually involved in metal reduction or their response was induced by increased iron concentrations.

Interestingly, no induction or repression was exhibited by the electron transport regulator *etrA* (data not shown). The deduced amino acid sequence of *etrA* shares a high degree of identity with FNR of *E. coli* (73.6%) and the analogous ANR protein of *Pseudomonas aeruginosa* (50.8%; Saffarini and Nealson, 1993). Microarray data suggested that, similar to *fnr*, the *S. oneidensis etrA* gene exhibited constitutive expression under all growth conditions tested (Kiley and Beinert, 1998), a pattern that one would expect if EtrA is indeed functionally similar to FNR. Mutant studies are underway to determine the role of *etrA* in *S. oneidensis* MR-1.

Genes involved in substrate and cofactor transport

Preliminary function assignments indicated that the majority of genes in this group are involved in siderophore receptor systems and substrate transport mechanisms. In contrast to *B. subtilis*, whose siderophore biosynthesis and acquisition genes were induced by anaerobiosis (Ye et al., 2000), the expression levels of *S. oneidensis* Fe(III)-transport genes were decreased under anaerobic conditions. The most highly repressed genes included those encoding alcaligin siderophore biosynthesis proteins (ORFs 207–209; 8- to 23-fold) and a heme-hemopexin utilization protein (ORF 870; 4- to 18-fold) as well as a putative outer membrane protein W precursor (ORF 3704; 4- to 11-fold) (Table 1). The lower transcription levels displayed under

Fe(III)-reducing conditions by most of the iron acquisition genes is most likely due to Fur (ferric uptake regulator)-mediated repression. *S. oneidensis* MR-1 contains a functional and structural homolog of the Fur protein, which acts as an iron-responsive transcriptional regulator (Thompson et al., in press).

Increases in mRNA abundance under anaerobic conditions were observed for genes involved in sulfate and thiosulfate transport and binding. These included *cysTWA* and *cysPU* (Sirko et al., 1995), which displayed a two- to fourfold, and two- to sixfold induction in expression, respectively. The transcription levels of two putative outer membrane proteins of unknown function (ORFs 2304 and 3850) also increased three- to fourfold and five- to ninefold under anaerobic conditions (Table 1). A gene encoding a periplasmic copper-binding protein (ORF 2482), which shares 40% amino acid sequence identity to the NosD protein from *P. stutzeri* (Zumft et al., 1990), exhibited approximately a three-fold increase in expression under all anaerobic conditions tested. Sequence analysis indicated that the putative *nosD* gene of *S. oneidensis* is most likely cotranscribed with *nosL, nosF*, and *nosY* genes, which were shown previously to be required for the assembly of the copper-containing active site in nitrous oxide reductase (Hoeren et al., 1993; McGuirl et al., 1998). Surprisingly, sequence analysis did not reveal the presence of a nitrous oxide reductase in the *S. oneidensis* genome, suggesting that the *nosLDFY* operon may be involved in the assembly of another copper-containing oxidoreductase that is not involved in denitrification. This conclusion is further supported by the observation that *S. oneidensis* MR-1 does not produce nitrous oxide or N_2 gas in a respiratory manner. While more than 98% of the nitrogen from nitrate or nitrite was transformed into ammonium, less than 0.5–2% of the nitrogen was detected as N_2O, and no significant amounts of N_2 were detected (A. Murray, unpublished results).

Induction of genes involved in drug resistance, biosynthesis, and other cellular functions

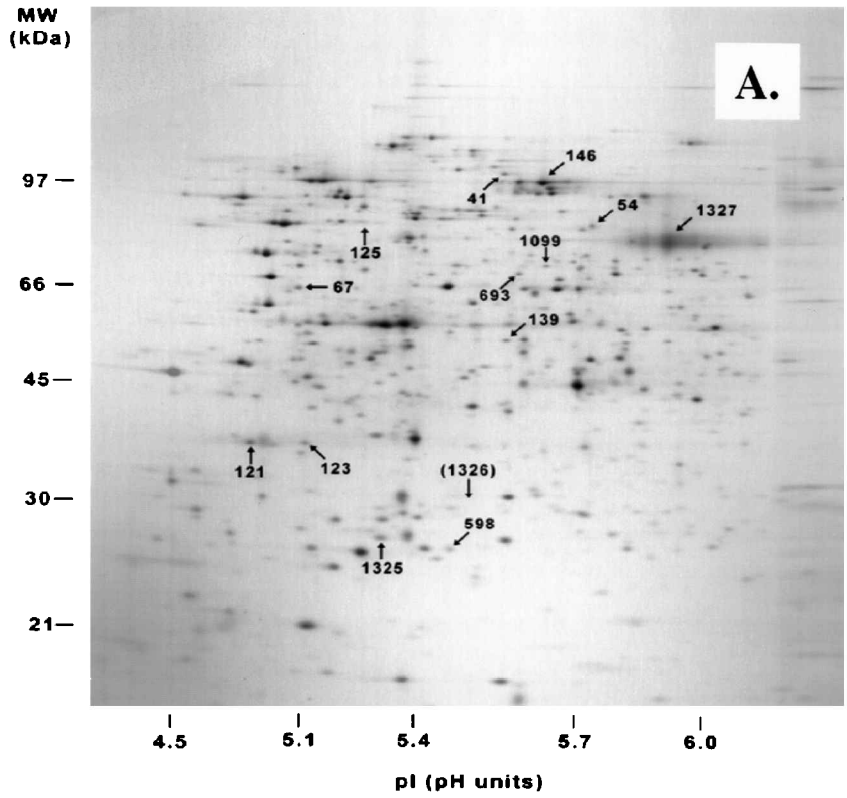
The mRNA synthesis for a number of penicillin-binding proteins was slightly increased under anaerobic growth conditions (Table 1). A probable membrane fusion protein involved in multidrug resistance (ORF 2520) was induced approximately sixfold under Fe(III)-reducing conditions, while the increase was marginal (less than twofold) under fumarate- and nitrate-reducing conditions. By contrast, the gene encoding the putative acriflavin resistance protein A precursor (ORF 2829) showed slight decreases in expression under all anaerobic respiratory conditions tested. It is unclear why genes with annotated functions in drug resistance displayed altered expression patterns under anaerobic respiratory conditions.

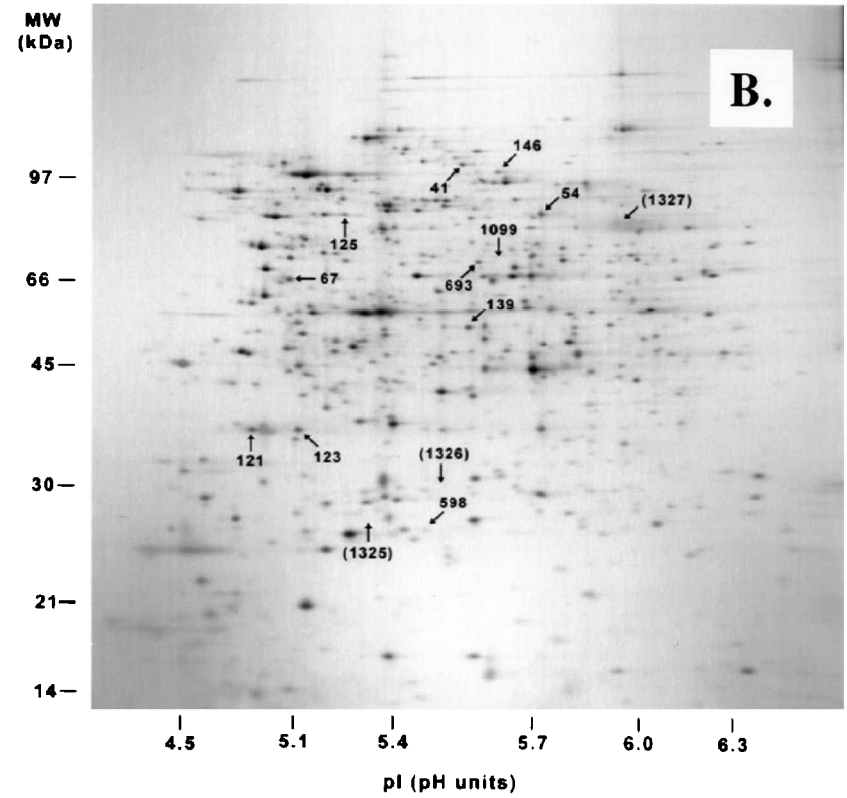
In addition to genes encoding drug resistance, microarray analysis indicated that a number of genes involved in biosynthesis and other diverse cellular functions exhibited altered expression levels in response to anaerobic respiration. Most notably, a putative siroheme synthase gene (ORF 930) displayed as much as a fivefold increase in transcript levels under nitrate-reducing conditions, with slightly lower induction ratios under conditions of Fe(III) and fumarate reduction (Table 1). Genes involved in molybdenum cofactor biosynthesis, *moaECA*, showed only minimal increases in mRNA synthesis. Homologs for *ccmHF* and *hemE* genes, which are involved in cytochrome *c* biogenesis and heme biosynthesis, respectively (Nishimura et al., 1993; Grovc et al., 1996), also were slightly induced in response to anaerobic growth conditions. Finally, the expression of genes encoding an anaerobic rNTP reductase and an anaerobic rNTP reductase activating protein was induced under all anaerobic growth conditions tested, while protease-encoding genes (ORFs 1164 and 1165) exhibited marginal decreases in mRNA abundance under fumarate and nitrate reduction and expression of a glutathione peroxidase-encoding gene (ORF 550) was repressed under all three anaerobic growth conditions.

Analysis of protein expression patterns using 2-D PAGE and mass spectrometry

To complement the microarray data, 2-D PAGE patterns of whole-cell lysates from aerobically grown *S. oneidensis* were compared to protein profiles obtained for cells grown anaerobically in the presence of fu-

FIG. 2. (*Next two pages.*) Two-dimensional polyacrylamide gel electrophoresis of whole-cell lysates of *S. oneidensis* MR-1 grown in LB medium under aerobic (**A**), fumarate- (**B**), Fe(III)- (**C**), and nitrate-reducing (**D**) conditions. Protein spots showing significant quantitative differences (at least $p < 0.05$) under aerobic, fumarate-, iron-, and nitrate-reducing conditions are denoted by arrows and numbers. Protein spots missing under aerobic conditions, but present under fumarate-, iron-, or nitrate-reducing conditions are indicated in parenthesis. The gel images are oriented with the isoelectric focusing dimension horizontal and the SDS-PAGE dimension vertical. The approximate pI is indicated along the horizontal axis. The approximate positions of the SDS-PAGE molecular mass standards are presented in kilodaltons along the vertical axis.


MW
(kDa)
A.
97
66
45
30
21
146
41
54
1327
1099
125
67
693
139
121
123
(1326)
598
1325
4.5
5.1
5.4
5.7
6.0
pI (pH units)


MW
(kDa)
B.
97
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54
(1327)
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125
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693
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(1325)
4.5
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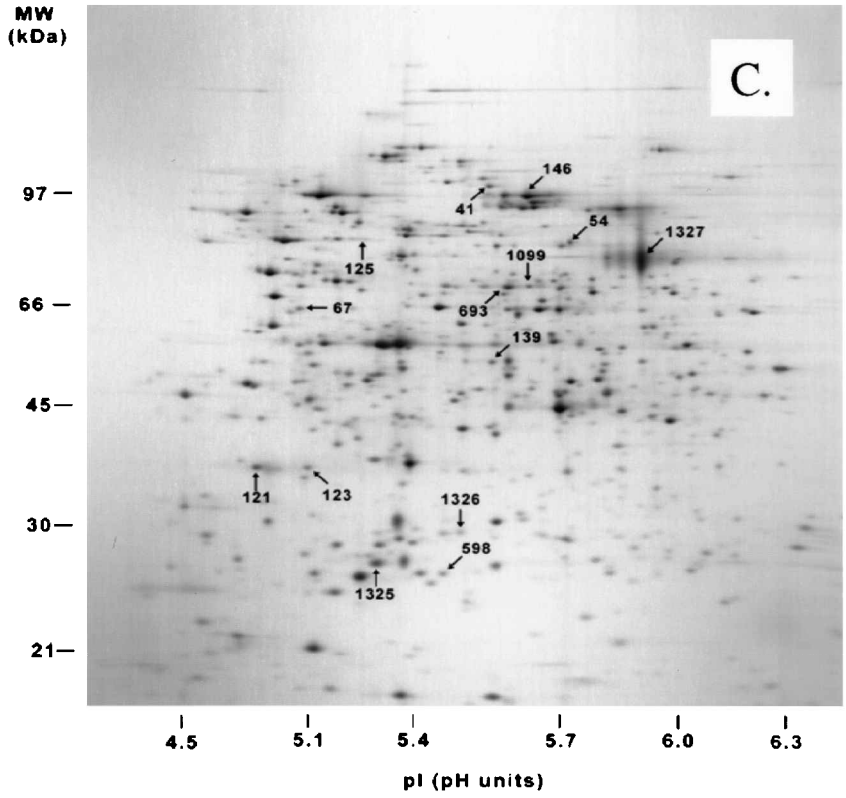
pI (pH units)



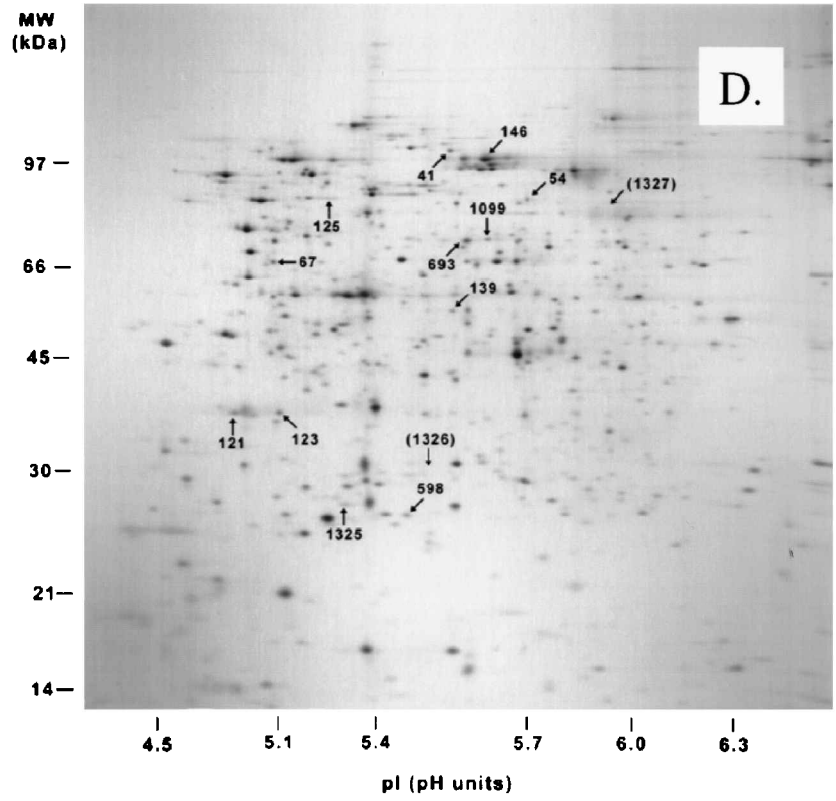


FIG. 2. (*Continued.*)

Table 2. Comparison of 2D-PAGE/Mass Spectrometry Data with mRNA Expression Ratios Derived from Microarray Hybridizations

Spot no.	ORF number/putative function[a]	Integrated density ratios[b]			mRNA expression ratios[c]		
		Fumarate-reducing conditions	Fe(III)-reducing conditions	Nitrate-reducing conditions	Fumarate-reducing conditions	Fe(III)-reducing conditions	Nitrate-reducing conditions
41	2013—DNA gyrase subunit β, *gyrB*	−2.56	−1.72	−2.63	−1.30 (±0.14)	1.35 (±0.08)	1.01 (±0.07)
	4153—Phenylalanyl-tRNA synthetase, β subunit *pheT*				N/A	N/A	N/A
	71—Formate acetyltransferase, *pflB*				−1.07 (±0.05)	1.51 (0.10)	1.08 (±0.05)
54	207—Alcaligin receptor protein, *alcC*	−3.45	−1.98	−1.75	−8.51 (±8.44)	−23.51 (±7.60)	−5.39 (±5.65)
	3956—Putative flavoprotein subunit, *sdhA*-like				−1.12 (±0.40)	1.06 (±0.05)	N/D
67	1553—Agglutination protein, *tolC*-like	−2.43	−2.57	−2.09	N/A	N/A	N/A
121	747—Outer membrane porin, *oprF*	−1.11	1.12	−2.34	N/A	N/A	N/A
123	343—Electron transfer flavoprotein, α subunit	−2.63	−1.85	−1.49	−2.79 (±1.45)	−8.01 (±4.24)	−2.03 (±0.24)
125	3403—Conserved hypothetical protein	−3.30	−2.89	−2.97	N/A	N/A	N/A
139	3959—Dihydrolipoamide succinyltransferase, *sucB*	−1.83	−1.46	−1.99	−3.31 (±1.61)	−2.38 (±0.21)	−2.61 (±1.18)
	3321—Glutamate-1-semialdehyde-2,1-aminomutase, *hemL*				N/A	N/A	N/A
146	71—Formate acetyltransferase, *pflB*	4.99	4.70	12.45	−1.06 (±0.05)	1.51 (±0.10)	−1.08 (±0.07)
598	3546—Uncharacterized ACR, YkgG family	1.92	3.00	3.21	N/A	N/A	N/A
693	3388—Prismane	1.36	17.14	12.39	1.78 (±0.06)	2.00 (±2.21)	55.59 (±0.74)
1099	3779—Phosphomannomutase, putative	5.01	6.57	6.92	−2.22 (±0.48)	−3.27 (±0.40)	−2.23 (±0.57)
1325	2142—Dihydroxy-2-butanone 4-phosphate synthase, *ribB*	>18	>49	>7	N/A	N/A	N/A
1326	1533—Conserved hypothetical protein	N/D	>10	N/D	N/A	N/A	N/A
1327	2987—Fumarate reductase, flavoprotein subunit, *frdA*	>50	>100	N/D	4.77 (±1.47)	1.41 (±0.19)	1.56 (±0.64)

[a]Sequence annotation was provided as a courtesy of TIGR (unpublished results). The ORF numbers provided here are for the purpose of tracking gene locations and annotation. The ORF numbers will change once the complete version of *S. oneidensis* MR-1 genome sequence annotation is released.

[b]For each targeted protein spot, relative integrated densities averaged from two to four replicate 2-D gels were compared by determining the ratio of protein abundance under fumarate-, Fe(III)-, and nitrate reducing conditions to that under aerobic growth conditions. Statistical analysis of the relative protein abundance was accomplished using a two-tailed *t* test. Only those proteins displaying quantitative differences with at least $p < 0.05$ were considered to differ significantly in abundance between the mutant and wild-type samples.

[c]Relative expression is presented as the ratio of the dye intensity of the anaerobically grown cultures to that of the aerobic reference. Positive values indicate induction fold under anaerobic conditions; negative values indicate the repression fold under anaerobic conditions (inverse negative value of the relative expression ratio). The data are mean values derived from eight to 16 total replicates. The standard deviation for each mean expression ratio is provided in parenthesis.

N/A, data not available, gene not represented on the partial microarray; N/D, not determined.

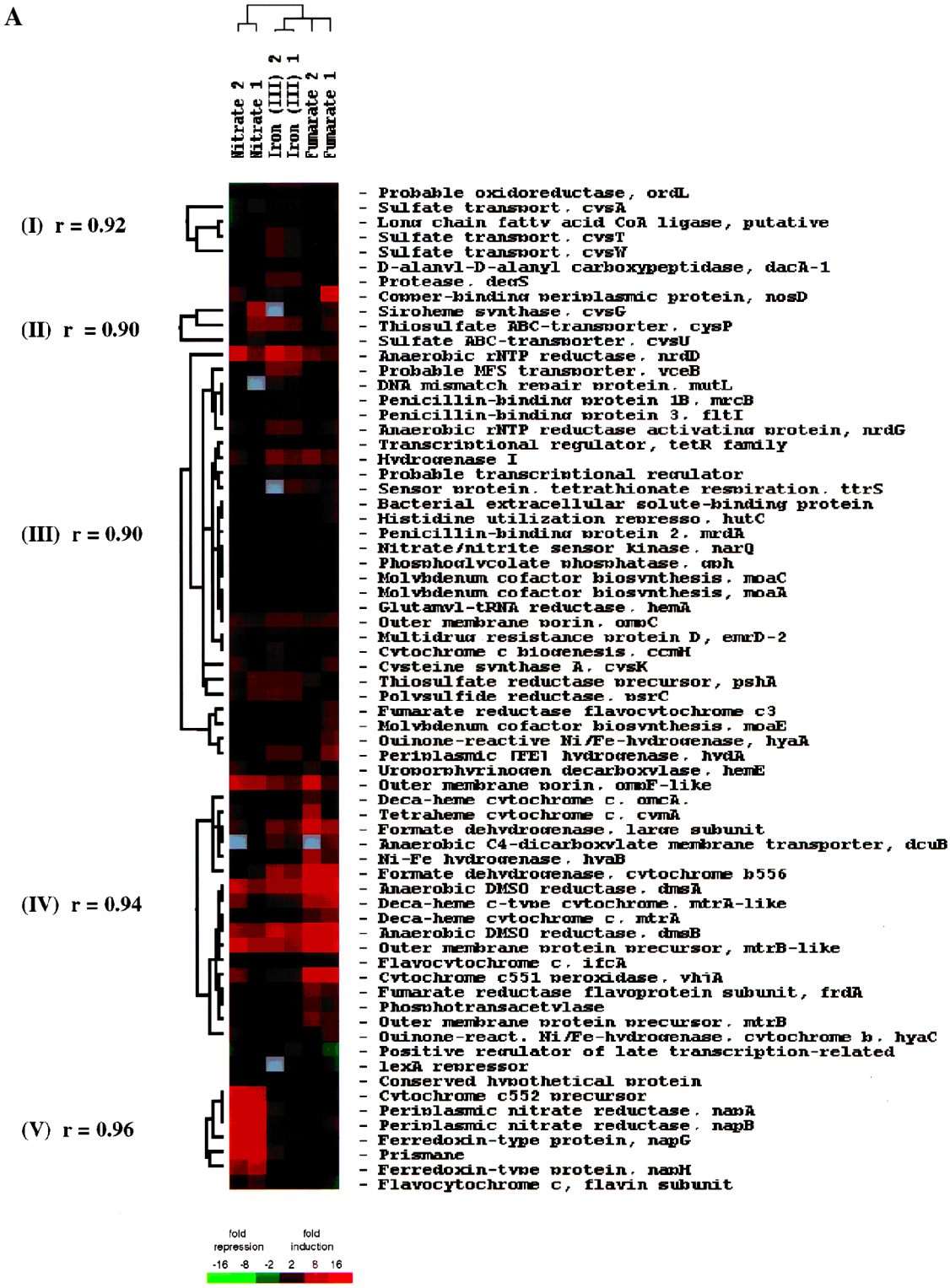


FIG. 3. (*Continued on next page*.) Hierarchical clustering of the 121 selected *S. oneidensis* MR-1 genes exhibiting altered expression patterns during the switch from aerobic to anaerobic growth. Each column indicates a separate biological replicate. (**A**) Clustering of genes displaying increased expression levels under anaerobic conditions. (**B**) Clustering of genes displaying decreased expression levels under anaerobic conditions. Red indicates upregulation, green represents downregulation, and gray indicates missing data points. The Pearson correlation coefficients (*r*) are displayed to the left of the nodes. We selected seven gene clusters (I–VII), for which the pairwise correlations were greater than 0.90.

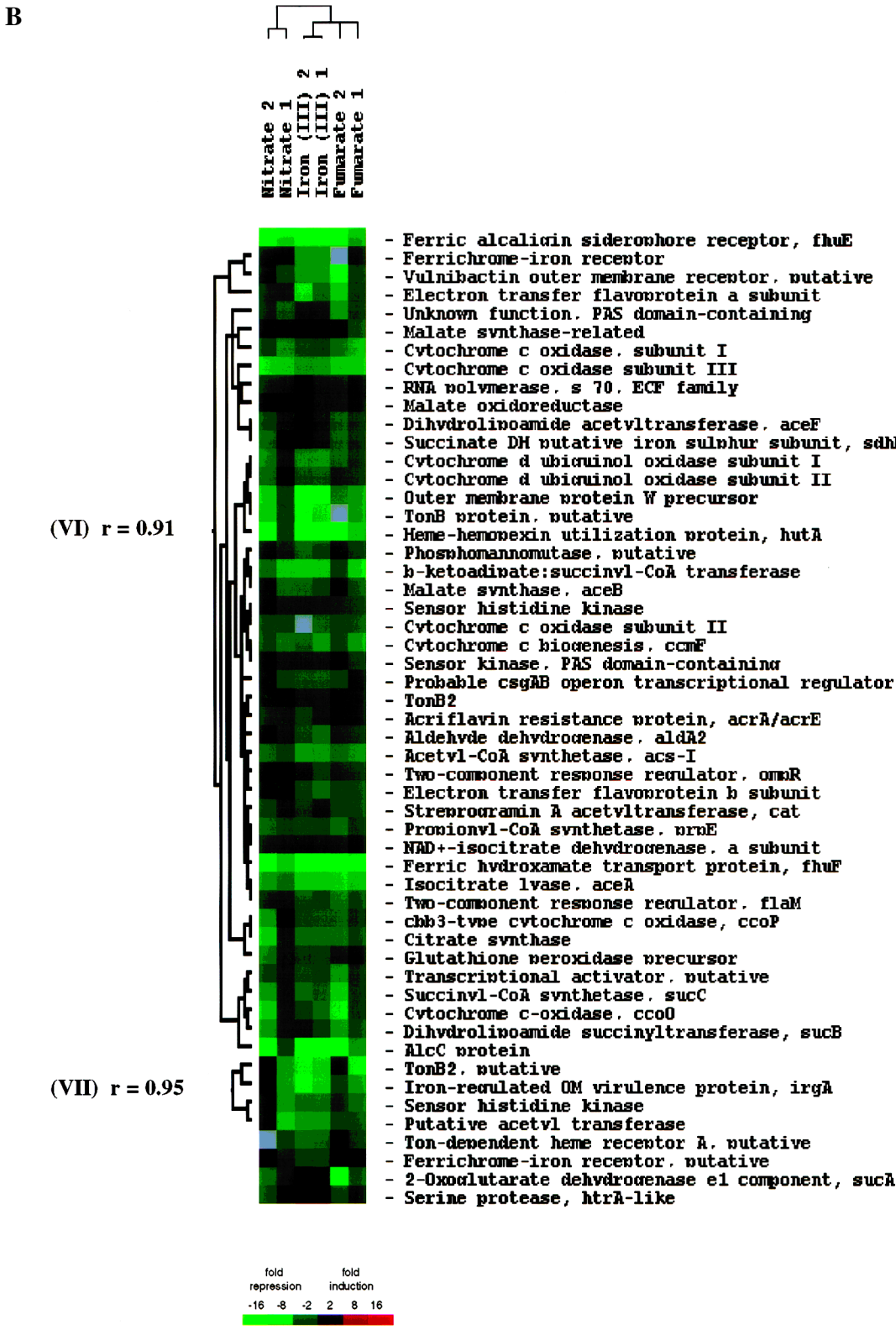


FIG. 3. (*Continued.*)

marate, ferric iron, and nitrate. Fourteen protein spots reproducibly showed significant ($p < 0.05$) differences in abundance under the various electron acceptor conditions (Fig. 2). A comparison of relative integrated densities revealed six proteins (labeled 41, 54, 67, 123, 125, and 139) that showed decreases in abundance under all three anaerobic conditions relative to aerobic growth (Table 2). Spots 67, 123, and 125 were unambiguously identified as an agglutination protein, electron transfer flavoprotein (α subunit), and a conserved hypothetical protein, respectively (Table 2). Spots 41, 54, and 139 contained multiple protein species based on tandem MS data. The expression patterns of four of these proteins, identified as AlcC (a protein putatively involved in alcaligin siderophore biosynthesis), electron transfer flavoprotein, and dihydrolipoamide succinyltransferase, were consistent with their mRNA levels as determined by microarray hybridization (Table 2). Genes encoding phenylalanyl-tRNA synthetase (β subunit), agglutination protein, the conserved hypothetical protein in spot 125, and glutamate-1-semialdehyde-2,1-aminomutase were not represented on the microarray. Prismane (spot 693), 3,4-dihydroxy-2-butanone 4-phosphate synthase (spot 1325), a conserved hypothetical protein (spot 1326), and fumarate reductase (spot 1327) exhibited particularly high abundance levels under Fe(III)-reducing conditions relative to the other respiratory conditions analyzed (Table 2). Additionally, prismane displayed a 12.4- and 1.4-fold increase in protein expression under nitrate- and fumarate-reducing conditions, respectively, compared to the level in aerobically grown cells. Microarray expression data indicated that transcript levels for the prismane-encoding gene under nitrate-reducing conditions correlated with the abundance of the gene product under the same growth conditions (Table 2). Not surprisingly, fumarate reductase also showed increased expression under fumarate-reducing conditions, whereas the protein was not detectable under nitrate respiration. While expression of the fumarate reductase-encoding gene was induced fivefold under fumarate-reducing conditions, transcript levels under anaerobic growth with Fe(III) were substantially lower (a 1.4-fold induction) in contrast to the protein abundance (Table 2).

Formate acetyltransferase (spot 146) showed the highest increase in abundance under nitrate-reducing conditions, but the mRNA abundance data for the gene did not correlate with the protein expression pattern. Similarly, the microarray-determined expression profile for the gene encoding a putative phosphomannomutase was not consistent with the expression profile of the protein, which displayed relatively high abundance under all three anaerobic conditions relative to aerobic growth. The inconsistency of mRNA and protein expression levels for these levels could be due to the different turnover rates of mRNA and protein molecules as well as posttranscriptional regulation (Anderson and Seilhamer, 1997).

One conserved hypothetical protein (spot 598) showed increased expression under both Fe(III)- and nitrate-reducing conditions, while the conserved hypothetical protein identified in spot 1326 displayed measurable, high abundance only under anaerobic respiration with ferric iron, suggesting that this protein may be specifically involved in Fe(III) reduction. Since neither one of these unknown genes were included in the microarray analysis, further expression and mutant studies are required to determine their involvement in the process of iron reduction.

Cluster analysis

To identify groups of genes exhibiting similar patterns of expression in response to changes in growth conditions, we performed a pairwise average-linkage clustering analysis (Eisen et al., 1998). Hierarchical clustering groups together genes of known similar function as well as provides means of obtaining leads to the function of unknown or poorly characterized genes (Eisen et al., 1998). Within 121 genes showing altered transcript levels in response to anaerobiosis, we identified seven clusters (I–VII) using a correlation coefficient cutoff value of 0.90 (Fig. 3). The majority of genes displaying increased expression levels under anaerobic conditions were represented by groups I–V. As expected, genes repressed under aerobic conditions were clustered together within groups VI and VII and included cytochrome *c* and *d* oxidases, intermediary carbon metabolism genes, and genes involved in iron acquisition and transport (Fig. 3B).

The highest induction ratios were displayed by genes putatively involved in nitrate reduction (group V, $r = 0.96$; Fig. 3A). It is noteworthy that *napBGHA* operon and cytochrome c_{552} clustered together with the gene encoding prismane, providing further evidence of the involvement of this iron-sulfur protein in nitrate respiration. Similarly, the DMSO reductase operon along with a gene cluster required for Fe(III) and Mn(IV)

reduction, which includes *omcA*, *mtrA*, and *mtrB*, fall into the same cluster as fumarate-inducible genes (group IV, $r = 0.94$). This finding appears to be in agreement with earlier observations that *S. oneidensis* MR-1 can reduce Fe(III) when grown on fumarate (Myers and Nealson, 1990). By contrast, we did not identify any highly expressed electron transport genes specific to Fe(III) reduction. However, a moderate induction (three- to sixfold) was observed under iron-reducing conditions for a number of putative drug resistance genes associated with the cell envelope (group III, $r = 0.90$; Fig. 3A). Previous biochemical studies of iron reduction in *S. oneidensis* MR-1 revealed that the Fe (III) reductase activity is found exclusively in the membrane fractions, with 54–56% localized in the outer membrane (Myers and Myers, 1993). Although no drug resistance proteins have been implicated in this process, our findings suggest a possible involvement of *S. oneidensis* MR-1 cell envelope proteins in Fe(III) reduction. Alternatively, the increased expression levels of these genes may be a consequence of stress-related response to increased intracellular iron concentrations. The existence of unknown genes required for metal reduction cannot be ruled out as well, especially since our study was limited to the partial representation of *S. oneidensis* genome on the microarray. Additional experiments will need to be conducted (e.g., mutagenesis, whole-genome microarray profiling, enzyme assays) in order to fully define the components and mechanisms of metal-reducing pathways.

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