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DNA Microarray Analysis of Nitrogen Fixation and Fe(III) Reduction in *Geobacter sulfurreducens*†

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A DNA microarray representing the genome of *Geobacter sulfurreducens* was constructed for use in global gene expression profiling of cells under steady-state conditions with acetate as the electron donor and Fe(III) or fumarate as the electron acceptor. Reproducible differences in transcript levels were also observed in comparisons between cells grown with ammonia and those fixing atmospheric nitrogen. There was a high correlation between changes in transcript levels determined with microarray analyses and an evaluation of a subset of the genome with quantitative PCR. As expected, cells required to fix nitrogen had higher levels of transcripts of genes associated with nitrogen fixation, further demonstrating that the microarray approach could reliably detect important physiological changes. Cells grown with Fe(III) as the electron acceptor had higher levels of transcripts for *omcB*, a gene coding for an outer membrane *c*-type cytochrome that is essential for Fe(III) reduction. Several other *c*-type cytochrome genes also appeared to be up-regulated. An unexpected result was significantly higher levels of transcripts for genes which have a role in metal efflux, potentially suggesting the importance of maintaining metal homeostasis during release of soluble metals when reducing Fe(III). A substantial proportion (30%) of significantly expressed genes during Fe(III) reduction were genes of unknown function or hypothetical proteins, suggesting differences in Fe(III) reduction physiology among microorganisms which perform this metabolic process.

Geobacter species represent a rare example in environmental microbiology in which microorganisms closely related to those which predominate in the environment and carry out environmental processes of interest can readily be cultivated in the laboratory (41). Molecular analyses, which avoid culture bias, have demonstrated that microorganisms in the family *Geobacteraceae* are the dominant dissimilatory metal-reducing microorganisms in subsurface environments in which organic contaminants are being degraded with the reduction of Fe(III) (57, 58, 62) and in aquatic sediments where dissimilatory metal reduction is important (63). Stimulation of dissimilatory metal reduction in subsurface environments to promote in situ uranium (44) and vanadium (55) bioremediation typically results in an enrichment of *Geobacteraceae*, which in some cases can account for as much as 80% of the groundwater microbial community (1, 27, 54). In addition to conserving energy from electron transfer to metals, *Geobacteraceae* can use electrodes as a terminal electron acceptor (4, 5, 29). *Geobacteraceae* specifically colonize graphite electrodes harvesting electricity from marine and freshwater aquatic sediments (4, 26, 65). If an electrode is poised at low potential, *Geobacter* species can accept electrons from the electrode and reduce more electropositive electron acceptors, such as nitrate and fumarate (22).

Despite the environmental significance of *Geobacter* species, little is known about their physiology (9). The sequencing of the complete genome of *Geobacter sulfurreducens* (46) and the development of a genetic system for this organism (14), as well as the potential to mass culture this organism for biochemical studies (45), have made *G. sulfurreducens* the organism of choice for more detailed physiological investigations of this genus. Here we report on the development of an additional tool for the study of the physiology of *G. sulfurreducens*, a whole-genome DNA microarray, and demonstrate how this tool has provided insights into environmentally relevant processes such as nitrogen fixation and growth with Fe(III) as the electron acceptor.

MATERIALS AND METHODS

Cell growth. *G. sulfurreducens* (ATCC 51573) was obtained from our laboratory culture collection. Cells were grown under strict anaerobic conditions at 30°C in chemostats, as previously described (18), with acetate (5 mM) as the electron donor and Fe(III) citrate (55 mM) or fumarate (27.5 mM) as the electron acceptor. Under these conditions, acetate is the substrate limiting growth. For growth in the absence of fixed nitrogen, the ammonium chloride (4.7 mM) was omitted from the medium and fumarate served as the electron acceptor. Cultures were maintained at a dilution rate of 0.05 h^{-1} for 5 culture vessel volumes to ensure that cells were at steady state prior to harvesting. Cells were harvested by centrifugation at an angular velocity of 3,150 relative centrifugal force for fifteen minutes at 4°C, and the cell pellet was flash-frozen in liquid nitrogen and then stored at −80°C prior to RNA extraction.

RNA isolation. In order to extract total RNA, cells were mechanically disrupted with a FastPrep instrument (Qbiogene) with Lysing matrix B (Qbiogene), and nucleic acids were extracted with TRIzol reagent (Invitrogen) (a monophasic solution of phenol and guanidine isothiocyanate). Any residual DNA was removed using RNase-free DNase according to manufacturer's instructions (Ambion). The treated RNA was subsequently cleaned and concentrated with RNeasy minicolumns (QIAGEN Inc.). The quality of total RNA was assessed by agarose-formaldehyde gel electrophoresis, and the concentration was determined with a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies).

DNA microarray transcriptional profiling. The DNA microarray consisted of 3,417 unique PCR products representing predicted coding sequences of the *G. sulfurreducens* genome. An additional 51 coding sequences are represented in

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TABLE 1. Significant GO terms and TIGR biological role categories from the EASE analysis

Expt	System	Gene category	EASE score
Iron reduction			
Up-regulated	TIGR role	Cellular processes; detoxification	1.27E−03
	GO biological process	Metal ion transport	2.99E−03
	GO biological process	Transport	3.76E−03
	TIGR role	Transport and binding proteins; cations and iron-carrying compounds	2.01E−03
Down-regulated	GO molecular function	Heat shock protein activity	1.43E−04
	GO biological process	Protein folding	1.82E−04
	TIGR role	Protein fate; protein folding and stabilization	3.49E−04
Nitrogen fixation			
Up-regulated	TIGR role	Central intermediary metabolism; nitrogen fixation	1.06E−11
	GO biological process	Nitrogen fixation	1.15E−11
	GO biological process	Nitrogen metabolism	5.25E−10
	TIGR role	Transport and binding proteins; anions	1.07E−06
	GO molecular function	Oxidoreductase activity, acting on reduced ferredoxin as donor, dinitrogen as acceptor	3.69E−05
	GO biological process	Molybdate ion transport	3.69E−05
	GO molecular function	Nitrogenase activity	3.69E−05
	GO molecular function	Oxidoreductase activity, acting on iron-sulfur proteins as donors	5.98E−05
Down-regulated	GO biological process	Oxidative phosphorylation, NADH to ubiquinone	2.04E−06
	GO molecular function	Oxidoreductase activity, acting on NADH or NADPH	6.11E−06
	GO molecular function	Oxidoreductase activity	1.00E−05
	GO molecular function	NADH dehydrogenase (ubiquinone) activity	1.42E−05
	GO molecular function	Sodium ion transporter activity	1.42E−05
	GO molecular function	Oxidoreductase activity, acting on NADH or NADPH, quinone, or similar compound as acceptor	1.42E−05
	GO molecular function	Carrier activity	2.00E−05
	TIGR role	Energy metabolism; electron transport	2.71E−05
	GO molecular function	Heat shock protein activity	4.12E−05
	GO molecular function	Hydrogen ion transporter activity	9.62E−05

duplicate. Two clustered regularly interspaced short palindromic repeat (CRISPR) regions (31) identified from genome analysis (46) were also represented by an additional 15 PCR products. Following amplification and purification, amplicons (reporters) were resuspended to a concentration of 100 to 200 nM in 50% dimethyl sulfoxide and printed onto UltraGaps aminosilane-coated slides (Corning Life Sciences) using an Intelligent Automation Systems array spotter. All reporters were printed a total of six times per slide. Postprinting, all slides were cross-linked using a Stratalinker UV cross-linker (Stratagene) and stored under vacuum until use.

Approximately 5 μg of total RNA was used for indirect labeling with either cyanine 3 or cyanine 5 (Cy3/Cy5) fluorescent dyes, leading to production of approximately 4 to 5 μg of cDNA with approximately 230 and 200 pmol, respectively, of dye molecule incorporated per microgram of cDNA synthesized for the nitrogen fixation and Fe(III) reduction experiments. Triplicate control and treatment chemostat cultures were extracted for each experiment so that extracted RNA could be paired to produce three biological replicates from which hybridizations could be repeated (technical replicates). Prehybridization of slides consisted of their immersion in a solution of 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS), and 1% bovine serum albumin for 45 min at 42°C, after which slides were washed and dried. Labeled cDNA was resuspended in a solution of 50% formamide, 5× SSC–0.1% SDS and allowed to hybridize for 18 to 20 h at 42°C under glass coverslips. Posthybridization, slides were washed two times for four minutes each in the following series of solutions: 1× SSC, 0.2% SDS; 0.1× SSC, 0.1% SDS; and 0.1× SSC. A final wash for 30 s at 0.05× SSC was completed prior to drying by centrifugation. Slides were promptly scanned at a 10-μm resolution using an Axon 4000B scanner with GenePix 4.0 software.

Processing of 16-bit TIFF images from hybridized arrays was performed with the TIGR TM4 package (www.tigr.org/software). Intensity values for Cy3 and Cy5 channels were obtained using TIGR-Spotfinder software. Normalization was performed with the LOWESS algorithm available in TIGR-MIDAS, using block mode and a smooth parameter of 0.33. All intensity values less than two times greater than background were removed from subsequent analysis, and replicate reporter intensities on one slide (one technical replicate) were reduced to a single value by computing the geometric mean. Six hybridizations were performed from each of three biological replicate chemostat pairs (control and treatment) for the Fe(III) condition and eight for the nitrogen-fixing condition. Half of the technical replicate dye labelings were dye swaps (flip dyes) performed as part of overall quality assurance (11).

To determine genes whose expression was significantly different from zero, significance analysis of microarrays (SAM) software (67) was employed using the one-class response with 1,000 permutations. The three biological replicates for each condition were analyzed individually. Significant genes were determined by setting the number of falsely called genes to less than one and choosing similar false discovery percentage medians for each biological replicate, resulting in similar total numbers of significant genes for output with a greater than 1.5-fold change in expression. At these levels, the q values (a measure of significance in terms of the false discovery rate) (64) for all biological replicates were less than 0.1, except for one biological replicate from the Fe(III) reduction experiment for which the q value was equal to 0.12. The intersections from the significant gene sets from each biological replicate were further analyzed. Significant genes present in two of three biological replicates based on SAM analysis determined that totals of 151 and 155 genes had significant changes in expression from the nitrogen fixation and Fe(III) reduction conditions, respectively.

EASE. Expression analysis systematic explorer (EASE) analysis was performed on the subset of genes determined to have significant changes in expression in at least two of three biological replicates from each experimental condition as identified by the SAM analysis. EASE uses a modified Fisher exact test (EASE score) to estimate the significance of classes of biological function present in a subset of significant genes relative to the total as represented on the array (30). TIGR role categories (www.tigr.org) and gene ontology (GO) terms (2) determined as part of the whole-genome annotation of *G. sulfurreducens* (46) were used as the biological classes examined for overrepresentation in the lists of significant genes and for all annotation referred to in this study. Only biological classes with EASE scores of $\leq 10^{-3}$ are reported (Table 1).

Measurement of relative transcript levels using real-time PCR. Total RNA isolated as described above was used as template for cDNA synthesis using random primers (Invitrogen, Carlsbad, CA) and Superscript II RNaseH$^-$ reverse transcriptase (Invitrogen). The resulting products were diluted 20-fold and used as a template for real-time PCR. Primer pairs unique to genes of interest were

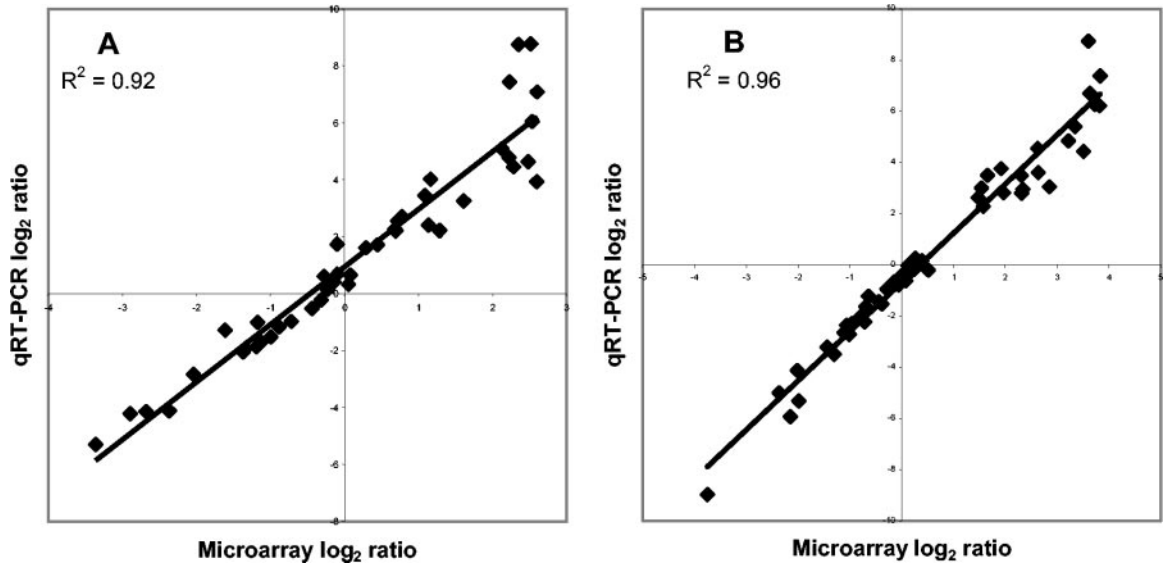

FIG. 1. Log_2 expression ratios from genes determined by quantitative real-time (qRT-PCR) versus microarray analysis across three biological replicates. Panel A represents expression ratios of genes from the Fe(III) reduction experiment. Panel B represents expression ratios of genes from the nitrogen fixation experiment. qRT-PCR results were produced by averaging triplicate measurements from three biological replicates. Microarray results were produced by averaging six (iron reduction) or eight (nitrogen fixation) technical replicates from three biological replicates. The linear relationship in panel A is given by $y = 2.0x + 0.93$ ($r^2 = 0.92$; $n = 45$ genes). The linear relationship in panel B is given by $y = 1.92x + 0.68$ ($r^2 = 0.96$; $n = 50$ genes).

designed using Primer3 software (59). Amplification was performed with SYBR Green PCR Master kit (Applied Biosystems). Real-time monitoring of fluorescence was performed during the cycling protocol according to manufacturer's instructions. Amplifications were performed in triplicate for each gene from each biological replication (Fig. 1). Totals of fifty and forty-five genes, respectively, were evaluated from the nitrogen fixation and Fe(III) reduction experiments (nitrogen fixation, 20 genes with >1.5-fold increased expression, 13 genes with 0 to 1.5-fold change, 17 genes with >1.5-fold decreased expression; Fe(III) reduction, 22 genes with >1.5-fold increased expression, 10 genes with 0- to 1.5-fold change, 13 genes with >1.5-fold decreased expression). A subset of eighteen genes was evaluated in each experimental condition and is listed in Table 1 along with primers used for their amplification. Expression levels were calculated using a method based on the change in threshold cycle as described by Wei et al. (69) (Fig. 1; Table 2).

ArrayExpress. All microarray data presented here are in accordance with the Microarray Gene Expression Data (MGED) Society's minimum information about a microarray experiment (MIAME) recommendations (6). Descriptions of the microarray experiments, quantitation data, and array design have been de-

TABLE 2. Genes whose expression levels were investigated by quantitative real-time PCR in both the nitrogen fixation and Fe(III) reduction experimental conditions

Locus identification no.	Common name	Primer sequence	
		Forward	Reverse
GSU0003	DNA gyrase, B subunit	AAGGGTGAAAAGGCAGGAGT	TCGTGGGTGTTGATGTTGTT
GSU0782	Nickel-dependent hydrogenase, small subunit	GGTGATCTGGCTTCACTTCC	TCCAGCGAAATCATGTCAAG
GSU0783	Nickel-dependent hydrogenase, iron-sulfur cluster-binding protein	CTACGGCGAGAAGGAAGTTG	CCCCTTGTAGATGGTGTGCT
GSU0784	Nickel-dependent hydrogenase, membrane protein	ACTCGGTCATGTTCGAGGTC	TAGAGCCAGTGGGACGATTC
GSU1305	Glu/Leu/Phe/Val dehydrogenase family protein	CCGAAGGGGTCAAGGTATTT	GCTTCTTCTCGGTGTCTTCG
GSU1331	Efflux transporter, RND family, MFP subunit	ATCGAAGGGCAGTACGTGAA	GAGGCAGAAGTCACCTCGAC
GSU1379	Ferric uptake regulation protein Fur	TCCATGATTACATCGCCAAG	CCCTGAGCTTGAGGTACAGC
GSU1470	Keto/oxoacid ferredoxin oxidoreductase, gamma subunit	CAACATCGTTGCTCTTGGTG	CTCGAAACCCATCTGGAAAG
GSU1496	Pilin domain protein	CCAACACAAGCAGCAAAAAG	GCAGCGAGAATACCGATGAT
GSU1836	Nitrogen regulatory protein P-II	CGTGGATTTTATCCCCAAGA	TGCGGATAACCTCTTCAACC
GSU2408	Heat shock protein, Hsp20 family	GGAACGGGTTCATGACAAAG	CATGAAGGCTGGCAAGGTAT
GSU2409	Heat shock protein, Hsp20 family	TGAAGAGACACGGTCAAACG	TGACATCCAGGGTTTCCTTC
GSU2490	Oxalate/formate antiporter, putative	GTTCCAGGCAGTCCTCATGT	CCCCAGTAATCCTTGCTGAA
GSU2751	C4-dicarboxylate transporter, anaerobic	ATCTCCGCCTTTGTGAAGAG	CAAGGTCGCTGGGATAGAAG
GSU2779	Transcriptional regulator, MerR family	GGCAGTGGACAAGGAGATGT	GGGAGAGCAAAAACTGATGG
GSU2804	Ferredoxin family protein	AGTTCGTCGTCTCCATCGAC	GAATCGTCTTCGTCCACCTC
GSU2821	Nitrogenase iron protein	AAGCTCGGCACTCAGATGAT	GCTTGTGCTCGGGAGAATAC
GSU3125	Alcohol dehydrogenase, zinc containing	TGGTGGTTTTCACCACCTCT	GTCGAGGATGAAGTCGAACG

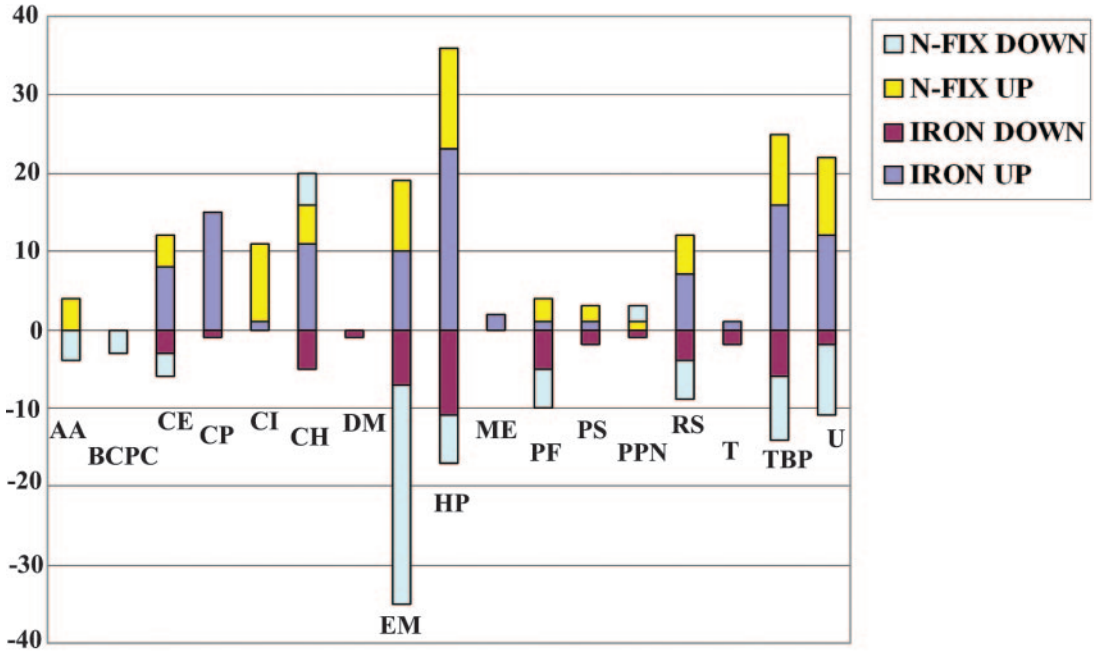

FIG. 2. A global view of relative changes in mRNA expression across the conditions of nitrogen fixation and Fe(III) reduction. The height of each bar represents the number of genes from either experimental condition whose expression was significantly elevated or diminished in a biological role category in each condition. The contribution of each gene from each experimental condition either elevated or diminished is represented by the area of the colored block within each bar. Positive values indicate genes with significantly increased expression; negative values indicate genes with significantly decreased expression. N-FIX DOWN, genes of decreased expression from the nitrogen fixation experiment; N-FIX UP, genes of increased expression from the nitrogen fixation experiment; IRON DOWN, genes of decreased expression from the Fe(III) reduction experiment; IRON UP, genes of increased expression from the Fe(III) reduction experiment. Biological role categories are abbreviated as follows: AA, amino acid biosynthesis and metabolism; BCPC, biosynthesis of cofactors, prosthetic groups, and carriers; CE, cell envelope; CP, cellular processes; CI, central intermediary metabolism; CH, conserved hypothetical proteins; DM, DNA metabolism; EM, energy metabolism; HP, hypothetical proteins; ME, mobile elements; PF, protein fate; PS, protein synthesis; PPN, purines, pyrimidines, nucleosides, and nucleotides; RS, regulatory functions and two-component signaling; T, transcription; TBP, transport and binding proteins; U, enzymes of unknown function.

posited into ArrayExpress (www.ebi.ac.uk/arrayexpress) and have been assigned accession numbers E-TIGR-81, E-TIGR-82, and A-TIGR-17.

RESULTS

Comparison with quantitative PCR. One test of the validity of the microarray results was to evaluate changes in transcript levels with an alternative method. Analysis of the expression of genes in both studies, covering the full range of differences in transcript levels, demonstrated that results from quantitative PCR were highly correlated with the microarray results, and the trend of expression patterns (decreased, minimal change, increased) was the same for all genes compared (Fig. 1).

EASE analysis results and role category distribution. The greatest proportion overall of significantly expressed genes were previously annotated as hypothetical genes (predicted coding regions with no homologs in other organisms) or enzymes of unknown function (Fig. 2). In each experimental condition, hypothetical genes were the largest category of significantly expressed genes, with the exception of down-regulated genes in the nitrogen fixation experiment. In this case, genes related to energy metabolism were the highest represented role category. The overrepresentation of nitrogen fixation genes in the up-regulated genes of significance from the nitrogen fixation experiment was expected and confirmed by EASE analysis. However, other results of EASE analysis provided new insight into the representation of biological classes (Table 1). GO terms (2, 46) representing biological processes and functions related to transport were significantly overrepresented in the genes with increased expression from the Fe(III) reduction experiment and in the genes with increased and decreased expression from nitrogen fixation. Protein folding and heat shock protein activity were also overrepresented in the subsets of down-regulated genes from each experimental condition. These results elucidate new biological classes of physiological response that have not previously been noted or analyzed in detail in this organism.

Nitrogen fixation. Comparison of transcript levels between cells grown with ammonium and cells that were required to fix atmospheric nitrogen further indicated that the microarray approach reliably detected changes in gene expression. Cells grown in the absence of ammonium had acetylene reduction rates of 0.012 nmol/h compared to 0.003 nmol/h in ammonium-grown cells, providing evidence that these cells were fixing nitrogen (10). The steady-state concentration of cells in the nitrogen-fixing chemostats (0.178 ± 0.011 mg/ml; mean ± standard deviation, $n = 3$) was significantly lower than that in chemostats provided with ammonium (0.45 ± 0.007 mg/ml), which is further consistent with the diversion of electron flow and energy to nitrogen fixation.

As expected, the primary differences in transcript levels be-

tween cells grown with and without ammonia were in genes associated with nitrogen fixation (Fig. 2). A total of 106 genes (59 up-regulated, 58 down-regulated) exhibited significant changes in expression based on the SAM analysis. This number increased to 151 (74 up-regulated, 77 down-regulated) when extended to those genes with significant changes in expression in at least two of the three biological replicates (see Tables S1 and S2 in the supplemental material). These include transcripts for more than 15 members of the nitrogen-regulated (Ntr) response (56), such as two P-II signal transduction homologs critical for disseminating the condition of cellular nitrogen levels to regulatory targets, nitrogen fixation-associated genes (*nif* genes) responsible for the fixation of nitrogen gas including genes encoding subunits of nitrogenase (16) and an uptake hydrogenase responsible for recycling hydrogen evolved as a by-product of nitrogen fixation (15, 70) (Table S1 in the supplemental material). An additional noteworthy finding was the increased transcription for 13 hypothetical genes and 10 genes of unknown function in at least two of three biological replicates of nitrogen-fixing cells (Fig. 2; Table S1 in the supplemental material).

Transcript levels for glutamate dehydrogenase were much lower in the nitrogen-fixing cells (>14-fold decrease). Glutamate dehydrogenase has a high K_m for ammonia and thus would only be expected to be functional during growth with exogenous ammonia (21, 56). There was also a notable decrease in many transcripts for energy metabolism genes (36 total), including a suite of genes associated with acetate oxidation via the tricarboxylic acid cycle and a putative NADH dehydrogenase (Table S2 in the supplemental material). Genes for a number of periplasmic and membrane-associated *c*-type cytochromes that are unique to *G. sulfurreducens* also appeared to be down-regulated (Table S2 in the supplemental material), but the physiological significance of this is unknown. Additional down-regulated genes included a suite of transporters including those involved in the transport of branched-chain amino acids and sodium-solute symporters (Table S2 in the supplemental material).

Fe(III) reduction versus fumarate reduction. Although physiological responses to nitrogen fixation have been studied in a number of microorganisms, differential gene expression in response to the use of Fe(III) as an electron acceptor has not been well studied. In all three chemostats, transcript levels for 44 genes were higher in *G. sulfurreducens* during growth on Fe(III) than with fumarate as the electron acceptor, whereas transcript levels for 27 genes were lower. When the criterion was two of three chemostats, 103 genes and five reporters representing the CRISPR2 region, whose physiology is unknown (31), appeared to be up-regulated, whereas 47 were apparently down-regulated (Fig. 2; Tables S3 and S4 in the supplemental material). As detailed below, one of the genes for which there were higher levels of transcripts during growth on Fe(III) (GSU2737) has previously been shown to be up-regulated during growth on Fe(III), while a second *c*-type cytochrome gene (GSU1334) with increased expression in this study has also been linked experimentally to a role in dissimilatory metal reduction (61). However, the differential transcript levels of the other significantly expressed reporters in this analysis have not been previously studied, and thus this is the first indication that their expression may be related to Fe(III) reduction (Tables S3 and S4 in the supplemental material). For example, the transcript levels of one other putative *c*-type cytochrome (GSU2808) whose physiological role is unknown, were up-regulated.

There were also higher levels of transcripts for cytochrome *d* ubiquinol oxidase. It would not be expected that an enzyme known in other organisms (33) for electron transfer to oxygen would need to be more highly expressed under the extremely anaerobic conditions found in the Fe(III)-reducing conditions of the Fe(III)-reducing chemostats, which contain high (ca. 40 mM) concentrations of Fe(II) and thus are even more protected from oxygen contamination than the fumarate-reducing cultures. It may be that although this gene is annotated as an oxidase (46), in *G. sulfurreducens* it plays a role in electron transfer to Fe(III). This hypothesis warrants further investigation.

At least 16 genes devoted to transport were up-regulated during growth with Fe(III) (Table 3; Table S3 in the supplemental material), a finding also considered significant based on results of the EASE analysis (Table 1). Of these, six are members of the resistance-nodulation-division (RND) superfamily of efflux transporters or their associated proteins (Table 3; Table S3 in the supplemental material) (35, 60, 66), including representatives of the transmembrane-spanning heavy metal efflux pump *czcA* family (GSU0830, GSU1332) and one gene coding for the membrane fusion protein of the *czcB* family (GSU0829) (20, 52). In contrast, transcript levels for other members of the *czcA* and *czcB* families in the *G. sulfurreducens* genome (GSU2135, GSU2136, GSU3400) (46) were comparable during growth on Fe(III) and fumarate. This suggests that these members of the *czc* families are paralogs (recently duplicated genes) with different physiological roles and regulation (36).

Two up-regulated *czc* family members (GSU0829, GSU0830) (Table 3; Table S3 in the supplemental material) are located physically next to one another on the *G. sulfurreducens* chromosome and appear to be coregulated. Another group of genes located contiguously on the *G. sulfurreducens* chromosome define an apparent 10-gene region of metal-responsive genes (GSU1330 to GSU1341) (Table S3 in the supplemental material). Genes in this region had among the highest transcript levels determined during growth on Fe(III), with seven members having a >5-fold increase in their transcription levels. This region includes five putative metal efflux and ABC transporter genes previously described (Table 3; Table S3 in the supplemental material), two hypothetical genes, two genes coding for membrane-associated proteins, and a gene coding for a *c*-type cytochrome (GSU1334) described above.

An additional region of up-regulated genes on the *G. sulfurreducens* chromosome was also elucidated when cells were grown with Fe(III). This region, defined by the genes GSU0975 to GSU0986 inclusive, contained seven up-regulated genes out of twelve total predicted genes (Table S3 in the supplemental material) and appears to represent a phage region in the genome (47). This region was bounded by homologs to bacteriophage-like tail proteins that were both up-regulated (GSU0975, GSU0986), including one with predicted lysozyme activity (GSU0986) (24). The ten predicted genes between the phage tail genes are all of unknown function. However, at least one of the genes that is apparently up-

regulated is related to a secreted protein (GSU0977), while a second gene with increased expression (GSU0983) bears homology to *vgr*-related proteins for which roles associated with ligand-binding proteins located on the bacterial surface or secretion have been suggested (68). The possibility of lysozyme activity, secretion, and cell surface associations suggests that this region could play a role related to transport and protein secretion, although the ultimate functions of the genes in this location remain to be determined.

In contrast to the increased gene expression of apparent metal efflux transporters during growth on Fe(III), the *dcuB* gene (GSU2751), which codes for the fumarate transporter in *G. sulfurreducens* (8, 46), was among the genes with the greatest decrease (>12-fold) in transcription (Table S4 in the supplemental material). A suite of genes devoted to regulation and signal transduction also exhibited significant differences in their levels of transcription. For cells grown with Fe(III), no less than five transcriptional regulators (GSU0267, GSU0770, GSU1268, GSU2698, GSU2779) showed increased levels of transcription (Table S3 in the supplemental material), including a homolog of the *merR* transcriptional regulator (GSU2779) that exhibited a >8-fold increase in expression. Two DNA-binding response regulators (GSU2287, GSU3118) that function as part of two-component signal transduction systems (25) were also significantly up-regulated (Table S3 in the supplemental material). Conversely, transcript levels for the ferric uptake regulator (*fur*) showed that *fur* was among the significant down-regulated genes during growth on Fe(III) as well as two response regulators (GSU0300, GSU2046) (Table S4 in the supplemental material). Genes for the production of flagella and pili, as well as several chemotaxis genes, also exhibited increased gene expression during growth on Fe(III) (Table S3 in the supplemental material).

DISCUSSION

In the present investigation, DNA microarray-mediated global gene expression profiling of *G. sulfurreducens* from two environmentally relevant physiological conditions was undertaken. Gene expression profiles in each condition revealed some expected results, particularly in the condition of nitrogen fixation. However, in each experiment and especially in the relatively much less well-studied condition of Fe(III) reduction, new insights into *G. sulfurreducens* physiology were revealed.

The increased expression of more than one P-II signal transduction homolog when fixing nitrogen suggests the ability of *G. sulfurreducens* to establish hierarchical cascades in response to nitrogen limitation (16). While the decreased expression of genes related to branched-chain amino acid transport may reflect the lack of these amino acids as nitrogen sources in the surrounding medium and a subsequent down-regulation of this nitrogen-scavenging system (56). The solutes transported by the members of the sodium solute symport family are currently unknown in *G. sulfurreducens*; however, candidates for symport can include sources of nitrogen such as amino acids and urea (32). Therefore, the down-regulation of these genes in nitrogen-depleted media would be consistent with the possibility that these nitrogen sources are their target solutes.

The finding of a large number of hypothetical genes and

TABLE 3. Transport proteins whose expression was significantly increased when *G. sulfurreducens* was grown with Fe(III) as electron acceptor versus fumarate as the electron acceptor[a]

Locus identification no.	Common name	Category		Mean $\log_2$ ratio	Fold change
		Main role	Subrole		
GSU1330[b]	Metal ion efflux outer membrane protein family protein, putative	Cellular processes/transport and binding	Detoxification/cations and iron-carrying compounds	2.52	5.74
GSU1332[b]	Heavy metal efflux pump, CzcA family	Cellular processes/transport and binding	Detoxification/cations and iron-carrying compounds	2.41	5.31
GSU0830[b]	Heavy metal efflux pump, CzcA family	Cellular processes/transport and binding	Detoxification/cations and iron-carrying Compounds	0.72	1.65
GSU0829[b]	Heavy metal efflux pump, CzcB family	Cellular processes/transport and binding	Detoxification/cations and iron-carrying compounds	0.71	1.64
GSU0264	Drug resistance transporter, Bcr/CflA family	Cellular processes/transport and binding	Toxin production and resistance	2.31	4.97
GSU3406	Amino acid ABC transporter, periplasmic amino acid-binding protein	Transport and binding proteins	Amino acids, peptides, and amines	2.43	5.37
GSU3404	Amino acid ABC transporter, ATP-binding protein	Transport and binding proteins	Amino acids, peptides, and amines	0.94	1.92
GSU3401	Branched-chain amino acid ABC transporter, periplasmic amino acid-binding protein, putative	Transport and binding proteins	Amino acids, peptides, and amines	0.78	1.71
GSU2490	Oxalate/formate antiporter, putative	Transport and binding proteins	Carbohydrates, organic alcohols	2.54	5.81
GSU2303	Na^+/H^+ antiporter family protein	Transport and binding proteins	Cations and iron-carrying compounds	1.28	2.43
GSU0844	Potassium uptake protein, Trk family	Transport and binding proteins	Cations and iron-carrying compounds	0.72	1.64
GSU1340	ABC transporter, permease protein	Transport and binding proteins	Unknown substrate	3.09	8.53
GSU1341	ABC transporter, ATP-binding protein	Transport and binding proteins	Unknown substrate	2.86	7.26
GSU1331[b]	Efflux transporter, RND family, MFP subunit	Transport and binding proteins	Unknown substrate	1.61	3.05
GSU0392[b]	Efflux transporter, RND family, MFP subunit	Transport and binding proteins	Unknown substrate	0.81	1.76
GSU1900	Transporter, putative	Transport and binding proteins	Unknown substrate	0.64	1.56

[a] Results are sorted first by main role and subrole and then in descending order by fold change.
[b] Members of the RND superfamily of efflux transporters.

genes of unknown function with increased expression during conditions of nitrogen fixation indicates that investigation into their roles may provide further insights into the physiology of *Geobacter* species in environments in which they are required to fix nitrogen. This is important because the growth and activity of *Geobacter* species may commonly be limited by the availability of fixed nitrogen in subsurface environments (3, 28). For example, *Geobacter* species highly expressed *nifD*, a key subunit of nitrogenase, in sediments from the Fe(III) reduction zone of a petroleum-contaminated aquifer and in organic-poor subsurface sediments amended with acetate to promote dissimilatory metal reduction (28).

As seen in previous studies (13), levels of transcripts of *omcB* (GSU2737) were higher in cells grown on Fe(III) than in cells grown on fumarate. *omcB* codes for an outer membrane *c*-type cytochrome that is required for Fe(III) reduction in *G. sulfurreducens* (37). Genes (GSU2733, GSU2739) that are associated with *omcB* in an operon (38) were also more highly expressed during growth on Fe(III). In contrast, levels of transcripts for *omcC* were not higher during growth on Fe(III). *omcC* (GSU2731) codes for another outer membrane *c*-type cytochrome that is 78% identical to *omcB* at the amino acid level (38, 46). However, deletion of *omcC* has no impact on Fe(III) reduction (38). These microarray results not only further confirm previous information on mechanisms for Fe(III) reduction, they also further demonstrate that the microarray approach can provide reliable information on gene expression under Fe(III)-reducing conditions and suggest that increased gene expression under these circumstances can, at least in some instances, be indicative of a role for that gene in Fe(III) reduction.

Increased transcription levels during growth on Fe(III) of a 10-gene region (GSU1330 to GSU1341) that includes apparent metal efflux genes and a putative *c*-type cytochrome (GSU1334) may be related to roles in metal homeostasis and possibly energy metabolism as well. Cell suspensions of a mutant in which this cytochrome gene was deleted were deficient in the reduction of Fe(III) and U(VI) oxides suggesting that this cytochrome, which is predicted to be located in the outer membrane, plays a role in dissimilatory metal reduction (61). Based on previous genome comparative analyses (46), this cytochrome (GSU1334) is currently unique to the *Geobacteraceae*, suggesting that the mechanism of Fe(III) reduction in *Geobacter* spp. may be different in comparison to other prokaryotes capable of metal reduction.

The role of these putative metal efflux systems and other transporters up-regulated during Fe(III) reduction has yet to be investigated in *G. sulfurreducens*. In *Ralstonia eutrophus*, the *czc* metal efflux system is a proton-driven export complex consisting of an inner membrane and periplasmic and outer membrane proteins (genes designated *czcA*, *czcB*, and *czcC*, respectively) that facilitates cell detoxification and heavy metal resistance to cobalt, zinc, and cadmium ions (52, 53). Homologs to the *czc* system in *Escherichia coli* have been linked to mediating resistance to copper and silver ions (19) and other members of the RND superfamily and their associated proteins have been linked to a role in metal homeostasis (60). The substrates carried by the up-regulated transporters in *G. sulfurreducens* are unknown; however, the results of this study suggest the importance of at least some of these transporters in cation and more specifically metal cation transport. Reduction of Fe(III) can result in the release of high concentrations of dissolved Fe(II), as well as trace metals that may be adsorbed to Fe(III) oxides (40). Thus, metal efflux proteins could be important in maintaining metal homeostasis in *G. sulfurreducens* under these conditions.

Previous analysis of the genome of *G. sulfurreducens* has suggested that its metabolism is highly regulated (46). Results from the present investigation continue to corroborate this finding. Of the transcriptional regulators identified with significant changes in expression during growth on Fe(III), two are members of regulatory families noted for responses to metal concentrations. One of the genes with the greatest increase in expression of all up-regulated genes identified in the Fe(III) study (Table S3 in the supplemental material) is a member of the *merR* family of transcriptional regulators. Members of this family have been noted as metalloresponsive regulatory genes in a number of prokaryotes (7, 34, 48). An important regulatory gene determined to be down-regulated during growth on Fe(III) was the *fur* transcriptional regulator (Table S4 in the supplemental material). This decrease in transcription is a likely response to the very high levels of dissolved iron in these cultures. The Fur protein controls the expression of genes involved in the uptake of iron for assimilatory purposes and represses its own expression (17, 23).

The apparent down-regulation of *dcuB* (GSU2751), the gene coding for the fumarate transporter in *G. sulfurreducens* (8) during growth on Fe(III), was consistent with its currently assigned function. For example, *G. metallireducens* lacks this gene and cannot grow with fumarate as an electron acceptor. However, when *dcuB* is introduced and expressed in *G. metallireducens*, it can grow as well as *G. sulfurreducens* on fumarate (8). Genes physically located on the *G. sulfurreducens* chromosome around *dcuB*, including those coding for a putative *c*-type cytochrome (GSU2748), a putative RNA methylase (GSU2749), and an enzyme of unknown function (GSU2750), all genes whose functions have yet to be investigated, also had lower transcript levels during growth on Fe(III), suggesting that these genes may instead have important roles in growth on fumarate.

As previously noted, genes for the production of flagella and pili, as well as several chemotaxis genes, appeared up-regulated during growth on Fe(III). Previous studies on the closely related organism, *Geobacter metallireducens*, demonstrated that in batch cultures, pili and flagella were specifically produced during growth on insoluble Fe(III) oxides, but not during growth on chelated Fe(III) (12). However, the actual signal inducing the expression of pilin and flagella genes was not determined. The slow growth of *Geobacter* species on Fe(III) oxide compared to growth on Fe(III) citrate (39, 43) suggests that the cells are energy limited when Fe(III) oxide is the electron acceptor, even when acetate is provided in excess in batch culture. Thus, the energy-limited condition of the cells that is induced during growth under acetate-limiting conditions in chemostats may have an influence on the expression of genes coding for flagella and pilin similar to that of growth on Fe(III) oxide in batch culture. Differences between growth on Fe(III) and fumarate might be expected, as the energetic yield on fumarate is at least threefold higher than during growth on Fe(III) (13). Alternatively, the regulation of the expression of

these genes may be different in *G. sulfurreducens* than in *G. metallireducens*. The higher levels of transcripts for some chemotaxis genes are consistent with previous studies with *G. metallireducens* which have indicated that chemotaxis may play an important role in aiding *Geobacter* species to locate sources of Fe(III) (12).

There were a substantial number of genes (23) annotated as hypothetical and as yet not observed in any other organism, as well as 12 enzymes of unknown function that appeared to be up-regulated during growth on Fe(III). The finding that these hypothetical genes are expressed is important because it demonstrates that most likely these are in fact genes with some apparent function in *G. sulfurreducens*. That they appear to be more highly expressed during growth on Fe(III) suggests that they may have some role in Fe(III) reduction. If so, it is not surprising that these genes have not been previously described in other microorganisms, because little is known about the mechanisms of electron transfer to Fe(III) (42) and mechanisms for Fe(III) reduction may be significantly different in different organisms (49–51).

In conclusion, we successfully used a DNA microarray to profile global gene expression patterns in *G. sulfurreducens* during responses to nitrogen fixation and growth with Fe(III) as an electron acceptor. Our microarray results correlated well with quantitative PCR results and were congruent with previously published investigations, especially those related to the relatively well-studied physiology of nitrogen fixation. Of greater significance was the determination of several *c*-type cytochromes with apparent roles in Fe(III) reduction and the increased role of transport during this condition, including the up-regulation of a suite of transporters that may be related to metal homeostasis. In addition, approximately 30% of the genes with significant changes in expression levels during conditions of Fe(III) reduction have no homolog in other prokaryotes or are of unknown function, suggesting important differences in metal reduction physiology in *G. sulfurreducens* compared to other microorganisms capable of this process.

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