APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 2002, p. 881–892 Vol. 68, No. 2
0099-2240/02/$04.00+0 DOI: 10.1128/AEM.68.2.881–892.2002

Transcriptional and Proteomic Analysis of a Ferric Uptake Regulator (Fur) Mutant of *Shewanella oneidensis*: Possible Involvement of Fur in Energy Metabolism, Transcriptional Regulation, and Oxidative Stress

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Received 29 June 2001/Accepted 19 November 2001

The iron-directed, coordinate regulation of genes depends on the *fur* (ferric uptake regulator) gene product, which acts as an iron-responsive, transcriptional repressor protein. To investigate the biological function of a *fur* homolog in the dissimilatory metal-reducing bacterium *Shewanella oneidensis* MR-1, a *fur* knockout strain (FUR1) was generated by suicide plasmid integration into this gene and characterized using phenotype assays, DNA microarrays containing 691 arrayed genes, and two-dimensional polyacrylamide gel electrophoresis. Physiological studies indicated that FUR1 was similar to the wild-type strain when they were compared for anaerobic growth and reduction of various electron acceptors. Transcription profiling, however, revealed that genes with predicted functions in electron transport, energy metabolism, transcriptional regulation, and oxidative stress protection were either repressed (*ccoNQ*, *etrA*, cytochrome *b* and *c* maturation-encoding genes, *qor*, *yiaY*, *sodB*, *rpoH*, *phoB*, and *chvI*) or induced (*yggW*, *pdhC*, *prpC*, *aceE*, *fdhD*, and *ppc*) in the *fur* mutant. Disruption of *fur* also resulted in derepression of genes (*hxuC*, *alcC*, *fhuA*, *hemR*, *irgA*, and *ompW*) putatively involved in iron uptake. This agreed with the finding that the *fur* mutant produced threefold-higher levels of siderophore than the wild-type strain under conditions of sufficient iron. Analysis of a subset of the FUR1 proteome (i.e., primarily soluble cytoplasmic and periplasmic proteins) indicated that 11 major protein species reproducibly showed significant ($P < 0.05$) differences in abundance relative to the wild type. Protein identification using mass spectrometry indicated that the expression of two of these proteins (SodB and AlcC) correlated with the microarray data. These results suggest a possible regulatory role of *S. oneidensis* MR-1 Fur in energy metabolism that extends the traditional model of Fur as a negative regulator of iron acquisition systems.

Shewanella oneidensis MR-1 (formerly *S. putrefaciens* MR-1), a gram-negative facultatively anaerobic bacterium, is capable of coupling the generation of energy to the reduction of insoluble ferric iron (Fe^{3+}), as well as other compounds (e.g., manganese, uranium, nitrate, fumarate, and dimethyl sulfoxide) in the absence of O_2. Despite extensive research on electron transport-linked Fe(III) reduction, very little is known about the genetic basis and regulation of iron transport and metabolism in *S. oneidensis*. Sequence determination of the 5-Mb genome of *S. oneidensis* MR-1 has been completed recently by The Institute for Genomic Research (TIGR) with the support of the U.S. Department of Energy, thus permitting the global prediction of gene function based on sequence homology. Sequence annotation of the *S. oneidensis* MR-1 genome revealed a putative *fur* (ferric uptake regulator) gene. The annotated biological function of this gene, however, has not been verified experimentally.

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Fur is an important global regulator that controls siderophore-mediated iron assimilation (11, 17, 27, 31) and modulates, at least in part, the expression of alternative sigma factor and activator genes (52, 53), oxidative stress-protective genes (32–34, 51), virulence-associated genes (14, 25, 44, 71), and acid tolerance genes (21). Homologs of the *fur* gene have been reported for a variety of bacteria, including *Escherichia coli* (31), *Vibrio cholerae* (42), *Vibrio anguillarum* (68), *Salmonella enterica* serovar Typhimurium (21), *Neisseria meningitidis* (66), *Neisseria gonorrhoeae* (9, 67), *Staphylococcus aureus* (72), *Bacillus subtilis* (12), and *Pseudomonas* species (56, 69). In *E. coli* and other bacteria, the Fur protein (molecular weight, 15,000 to 17,000) functions as an iron-responsive repressor that utilizes Fe(II) as a cofactor and binds to specific sequence elements in the target promoters of iron-regulated genes, resulting in the transcriptional repression of these genes in iron-replete environments (5, 14, 19). In response to iron limitation, Fur no longer binds to the operator site, and transcription from target promoters resumes. In *E. coli*, the operator site, or so-called Fur box, is defined by the 19-mer palindromic consensus sequence GATAATGATAATCATTATC (19). A study by Ochsner and Vasil (53) revealed that 10 perfect base pair

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
E. coli S17-1/λ_{pir}	S17-1 derivative, RK2 *tra* regulon, *pir*, host for *pir*-dependent plasmids	37
S. oneidensis		
MR-1	Lake Oneida isolate	49
DSP10	Spontaneous mutant of MR-1, Rif^r	D. Lies, Caltech
FUR1	*fur* mutant derived from DSP10; *fur*::pKNOCK-Km^r	This work
Plasmid pKNOCK-Km^r	pBSL63 derivative carrying RP4 *ori*T and R6K γ-*ori*, Km^r	1

matches (53% identity) with the consensus sequence were necessary for a functional Fur-binding operator site in *P. aeruginosa* PAO1.

In contrast to other bacteria with well-characterized *fur* genes, *S. oneidensis* MR-1 uses iron for both the biosynthesis of cellular enzymes and macromolecules (assimilatory processes) and energy production (dissimilatory processes) (46, 50). To examine the importance of Fur in regulating gene expression in a dissimilatory metal-reducing bacterium, an *S. oneidensis* strain (FUR1) harboring an insertional disruption in the *fur* gene was created and then analyzed using DNA microarrays consisting of 691 open reading frames (ORFs) and two-dimensional (2-D) gel electrophoresis in conjunction with mass spectrometry. Besides the expected derepression of iron siderophore biosynthesis and receptor genes in FUR1, the *fur* mutation affected the transcription of a number of genes involved in electron transport systems, energy metabolism, and regulation as well as a putative Fnr-like regulatory gene, *etrA* (encoding electron transport regulator A). Physiology studies, however, revealed no substantial difference between the wild type and the *fur* mutant in the ability to grow and utilize different electron acceptors under anaerobic conditions. While the findings reported here support previous descriptions of Fur as a negative regulator of iron acquisition genes, this study also suggests that *S. oneidensis* Fur plays a role in the coordinate regulation of energy metabolism.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. A list of all bacterial strains and plasmids used in this study is given in Table 1. *S. oneidensis* and *E. coli* strains were grown in Luria-Bertani (LB) medium (59) at 30 and 37°C, respectively. When needed, the growth medium was supplemented with antibiotics at the following concentrations: for *E. coli* and *S. oneidensis*, 50 and 25 μg of kanamycin per ml, respectively; and for *S. oneidensis*, 10 μg of rifampin per ml. The suicide vector pKNOCK-Km^r has been described elsewhere (1).

Disruption of the *fur* locus in *S. oneidensis*. The putative *fur* gene was inactivated by integration of the suicide plasmid pKNOCK-Km^r (2 kb in size) into the chromosomal *fur* locus. An internal fragment (179 bp) of *fur* was amplified by PCR using the primers 5543IM-F (5′-TGCAAGGACCTGAAAACC-3′) and 5543IM-R (5′-CTGAGTCGATAACTCGAATACG-3′) and purified using the QIAquick PCR purification kit (Qiagen, Chatsworth, Calif.). The amplified fragment was cloned into the *Sma*I site of plasmid pKNOCK-Km^r using the Perfectly Blunt cloning kit (Novagen, Madison, Wis.) according to the manufacturer's instructions, and the resulting construct, *fur*::pKNOCK-Km^r, was introduced into competent *E. coli* S17–1/λ_{pir} cells by electroporation. Transformants were screened for the correct recombinant plasmid using the Fast-Link screening kit (Epicentre Technologies, Madison, Wis.). For conjugal transfer of the suicide plasmid construct, *E. coli* S17–1/λ_{pir} cells harboring the *fur*::pKNOCK-Km^r plasmid were used in mating experiments with strain DSP10, a spontaneous rifampin-resistant derivative of *S. oneidensis* MR-1. *E. coli* transformants and DSP10 cells were grown separately in LB medium overnight, washed in fresh medium, and mixed in a 1:1 ratio by being spotted onto 0.2-μm-pore-size Millipore membrane disks. Following a 6-h incubation at room temperature, cells were removed from the filter disks, resuspended in medium, and plated onto LB agar plates supplemented with kanamycin (25 μg ml^{-1}) and rifampin (10 μg ml^{-1}).

Correct integration of the pKNOCK-Km^r suicide vector into the *fur* locus was verified by PCR amplification and reverse transcription-PCR (RT-PCR) analysis. PCR confirmation was accomplished by comparing the sizes of the products amplified from wild-type and mutant DNAs by using *fur*-specific primers that flanked the pKNOCK-Km^r insertion sites. The forward external primer 5543F (5′-GGTTTGAAAATCACCCTGC-3′) and the reverse external primer 5543R (5′-ATTGTACTTACTGGCAATCTCG-3′) were used. For RT-PCR, 2 μg of DNase I-treated total RNA from wild-type and *fur* mutant cells served as the template for cDNA synthesis in a reverse transcription reaction mixture containing 10 μM primer 5543R, 4 μl of 5× First Strand buffer (Gibco BRL, Gaithersburg, Md.), 1 μl of 0.1 M dithiothreitol (Gibco BRL), 10 mM deoxynucleoside triphosphates, and 200 U of Superscript II RNase H$^-$ reverse transcriptase (Gibco BRL). Reaction mixtures (total volume of 17 μl) were incubated at 37°C for 1 h. Two microliters of each reverse transcription product was used in PCR amplification with the primers 5543IM-F and 5543R.

Siderophore production and anaerobic growth on various electron acceptors. Siderophore biosynthesis and secretion by FUR1 were compared with those by the wild-type strain in LB medium with or without the addition of 50 μM $FeCl_3$. Cultures of these strains were grown aerobically to stationary phase (optical density at 600 nm [OD_{600}] = 4) at 30°C. Cell-free culture supernatants (500 μl) were mixed with an equal volume of chrome azurol S assay solution prepared as described previously (61) and incubated at room temperature for 2 h before the absorbance at 630 nm was measured. Siderophore production was calculated as the ratio of supernatant A_{630} to control (uninoculated medium) A_{630} from dilutions giving a linear range of absorbance.

Growth on different electron acceptors under anaerobic conditions was determined essentially as described previously (7). Briefly, wild-type (DSP10) and mutant (FUR1) strains of *S. oneidensis* were grown anaerobically in a Coy anaerobic chamber using LM medium (48) supplemented with 20 mM lactate as the electron donor and MnO_2 (500 μM), $Fe(OH)_3$ (500 μM), Fe(III) citrate (10 mM), thiosulfate (10 mM), fumarate (10 mM), dimethyl sulfoxide (DMSO) (10 mM), trimethylamine *N*-oxide (TMAO) (10 mM), anthraquinone-2,6-disulfonic acid (AQDS) (5 mM), nitrate (2 mM), nitrite (2 mM), or sulfite (2 mM) as the electron acceptor. Growth was assessed spectrophotometrically at 600 nm using end point growth determinations. Rates of metal reduction were measured as described previously (7). Short-term anaerobic growth of the wild-type and FUR1 strains of *S. oneidensis* was compared using LB broth supplemented with 20 mM sodium lactate and 8 mM nitrate or 10 mM fumarate. Growth was monitored by measuring the OD_{600} at 30-min intervals over a 6-h period and at 24 h.

PCR amplification and microarray construction. Because sequence determination of the *S. oneidensis* MR-1 genome was not finalized at the time of this study, partial genome microarrays were constructed that contained 691 PCR-amplified MR-1 ORFs putatively involved in energy metabolism, transcriptional regulation, adaptive responses to environmental stress, iron acquisition, and transport systems (for a detailed listing, see our website at http://www.esd.ornl.gov/facilities/genomics/partial_microarrays.html). PCR primers used to amplify probable genes from *S. oneidensis* MR-1 were designed using the computer program Primer 3 (Whitehead Institute) with genome sequence information provided as a courtesy of TIGR (John Heidelberg, personal communication) and then synthesized at Stanford University. To simplify PCR amplifications, all primers were designed to have similar melting temperatures. Primer sequences were searched against the genome database for *S. oneidensis* MR-1 with FASTA to evaluate the potential for cross-amplification among different homologous genes. Amplified DNA fragments were considered correct if PCRs contained a single product of the expected size as determined by agarose gel electrophoresis. For purposes of quantification, the following control DNA samples were also included on the array: (i) a set of three serial dilutions of *S. oneidensis* MR-1 genomic DNA spotted onto each corner of each quadrant of the array to allow two-channel normalization of the fluorescence over a range of signal intensities, (ii) pUC19 plasmid as a negative control, (iii) non-*S. oneidensis* DNA from yeast as an additional negative control, (iv) internal standards derived from five yeast genes that were cloned into vectors to allow transcription from a T7 promoter, and (v) blank control spots.

PCR products (concentrations ranging from 100 to 300 ng μl^{-1}) prepared in 50% DMSO (Sigma Chemical Co., St. Louis, Mo.) were spotted onto glass CMT-GAPS slides (Corning, Corning, N.Y.) with ChipMaker 3 pins (Telechem International, Sunnyvale, Calif.) using a PixSys 5500 robotic printer (Cartesian Technologies, Inc., Irvine, Calif.) under conditions of 62% relative humidity. PCR products representing 691 different ORFs were spotted in four replicates on a single slide. Postprocessing of the microarray slides was carried out according to the protocol of the manufacturer (Corning).

RNA isolation and preparation of fluorescein-labeled cDNA. Total cellular RNAs from wild-type and *fur* mutant strains of *S. oneidensis* grown in the presence or absence of 50 μM ferric citrate were isolated using the TRIzol Reagent (Gibco BRL) according to the manufacturer's instructions. RNA samples were treated with RNase-free DNase I (Ambion, Inc., Austin, Tex.) to digest residual chromosomal DNA and then purified with the Qiagen RNeasy Mini kit prior to spectrophotometric quantitation at wavelengths of 260 and 280 nm.

Fluorescently labeled cDNA copies of total cellular RNA extracted from wild-type and mutant cells were prepared by incorporation of fluorescein-labeled nucleotide analogs during a first-strand reverse transcription reaction. RNA from the wild-type strain was fluorescently labeled with Cy5 (or Cy3), and that from the mutant was labeled with Cy3 (or Cy5). Two sets of duplicate reactions were carried out in which the fluorescent dyes were reversed during cDNA synthesis to minimize gene-specific dye effects. Each 30-μl labeling reaction mixture contained 10 μg of total cellular RNA; 9 μg of random hexamer primers (Gibco BRL); 10 mM (each) dATP, dGTP, and dTTP; 0.5 mM dCTP; 3 μl of 0.1 M dithiothreitol; 40 U of RNase inhibitor (Gibco BRL); 1 mM either Cy3-dCTP or Cy5-dCTP (Perkin-Elmer/NEN Life Science Products, Boston, Mass.); and 200 U of Superscript II RNase H^- reverse transcriptase in 1× First Strand buffer. The reverse transcription reaction was allowed to proceed for 2 h at 42°C. The labeled cDNA probe was treated with 1 N NaOH to remove residual RNA, purified using a Qiagen PCR purification column, and concentrated in an SC110 Speedvac (Savant Instruments, Inc., Holbrook, N.Y.).

Microarray hybridization and scanning. Gene expression analysis was performed using four independent microarray experiments, with each slide containing four replicate arrays (a possible total of 16 data points per gene). The two labeled cDNA pools (wild type and mutant) to be compared were mixed and hybridized simultaneously to the array in a solution containing 3× saline sodium citrate (SSC) (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.3% sodium dodecyl sulfate (SDS), and 24 μg of unlabeled herring sperm DNA (Gibco BRL). Hybridization was carried out under a 22- by 40-mm Hybrislip (Sigma) in a waterproof CMT-slide chamber (Corning) submerged in a 65°C water bath for 12 to 15 h. Following hybridization, slides were washed with 1× SSC–0.2% SDS and 0.1× SSC–0.2% SDS for 5 min each at ambient temperature and then with 0.1× SSC for 30 s at ambient temperature prior to being air dried. Microarrays were scanned using the confocal laser microscope of the ScanArray 5000 Microarray Analysis System (GSI Lumonics, Watertown, Mass.) at a resolution of 5 μm per pixel. Scanning parameters (laser power and photomultiplier tube or PMT gain) were adjusted, so that overall intensities in both fluorescence channels were relatively equal and few spots were saturated.

Quantitative analysis of hybridization intensities and normalization. To determine fluorescence intensity (pixel density) and background intensity, 16-bit TIFF scanned images were analyzed using the software ImaGene version 3.0 (Biodiscovery, Inc., Los Angeles, Calif.). Prior to channel normalization, microarray outputs were first filtered to remove spots of poor signal quality by excluding those data points with a mean intensity of less than two standard deviations above the overall background for both channels (35). Channel normalization was accomplished using the geometric mean normalization algorithm (N. Morrison et al., Nature Genetics Microarray Meeting, Scottsdale, Ariz., 1999). Briefly, this included calculating the trimmed geometric mean (TGM) for natural log-transformed signal intensities and then calculating $(\ln[X] - \mathrm{TGM}[X]) \times (\mathrm{SD}_{\mathrm{TGM}\ X})^{-1}$, where X represents the signal intensity and SD is the standard deviation. The values were converted from log space, and fluorescence ratios (e.g., Cy5/Cy3) were determined. Log-transformed fluorescence ratios for each experiment were inspected to determine experimental quality and distribution of the ratios. The TGM normalized ratios were averaged among arrays with the fluorophores reversed (40, 73). The expression of a gene was considered significantly different if the ratio of the two fluorescent signals was greater than 2 (60).

2-D PAGE of whole-cell lysates. Cell pellets of *S. oneidensis* wild-type and *fur* mutant strains were mixed separately with 5 volumes of a solution containing 9 M urea, 2% (vol/vol) 2-mercaptoethanol, 2% (vol/vol) pH 8 to 10 ampholytes (Bio-Rad, Hercules, Calif.), and 4% (vol/vol) Nonidet P-40. The lysates were centrifuged for 10 min at 435,000 × *g* in a Beckman TL100 ultracentrifuge to sediment all particulates. Protein concentrations were determined using a modified Bradford method (57). Supernatants were stored at −70°C until analyzed by 2-D gel electrophoresis.

Isoelectric focusing gels were cast as previously described by Anderson and Anderson (2), using a 2:1 mixture of pH 5 to 7 and pH 3 to 10 ampholytes (Bio-Rad). 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 the application of statistical tests. Following isoelectric focusing, the gels were equilibrated in a buffer containing SDS as described previously (54). The second-dimension slab gels were cast using a linear gradient of 10 to 17% polyacrylamide. The equilibrated tube gels were secured to the slab gels using agarose, and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described previously (3). The following proteins (Sigma) were added as molecular weight standards: 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 (23).

Image acquisition and analysis of 2-D gels. The 2-D gel images were digitized using an Eikonix 1412 charge-coupled device scanner interfaced with a VAX 4000 to 90 workstation. The images were then processed, and parameter lists (spot files) were generated using the Tycho II software developed at Argonne National Laboratory (4). An *S. oneidensis* wild-type spot file was copied to serve as the reference pattern for the experiment. On average, over 1,000 spots were detected on each 2-D gel image. Of these, approximately 800 of the most reproducible spots were included in the data analysis. All spot files (two or three two-dimensional gel patterns per cell sample) were matched to the reference pattern 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 Student *t* test as described previously (24). Only those proteins showing quantitative differences with at least a probability (P) of less than 0.05 were considered to differ significantly in abundance between the wild-type and mutant samples.

Protein identification by mass spectrometry. One hundred fifty to 200 micrograms of protein was separated by isoelectric focusing. After separation in a second SDS-PAGE dimension, the proteins were detected by staining the gel with Coomassie blue R250 for 18 h. Protein spots to be identified were excised from one to five replicate gels (the number of spots required varied with the abundance of individual proteins) and processed for mass spectrometric analysis by following the procedure developed by Shevchenko et al. (62). Briefly, excised spots were reduced at room temperature with tris(2-carboxyethyl) phosphine (Pierce, Rockport, Ill.), alkylated with iodoacetamide (Sigma), and digested in situ with modified trypsin (Promega Corp., Madison, Wis.). Peptides were extracted from the gel by changes of 25 mM ammonium bicarbonate and 5% formic acid in 50% acetonitrile and then analyzed by micro-liquid chromatography-electrospray ionization tandem mass spectrometry (micro-LC-ESI-MS/MS).

For micro-LC-ESI-MS/MS, samples were loaded onto an in-house-constructed fritless 365- by 100-μm fused silica capillary column (22) packed with 5-μm Zorbax XDB-C18 packing material (Agilent Technologies, Palo Alto, Calif.) at a length of 7 to 8 cm. The flow rate at the tip was controlled to 200 to 300 nl/min using a precolumn splitter. The tryptic peptides were separated with a 30-min linear gradient of 0 to 60% solvent B (80% acetonitrile–0.02% heptafluorobutyric acid) and then entered an LCQ ion-trap mass spectrometer (Thermo Finnigan, San Jose, Calif.). Tandem mass spectra were automatically collected in the data-dependent mode during the 30-min LC-MS runs. Obtained MS/MS spectra were then directly subjected to SEQUEST (20) database searches without the need for manual interpretation. SEQUEST identified proteins in a spot by correlating experimental MS/MS spectra to protein sequence in the *S. oneidensis* MR-1 database (41).

Computer analyses. The sequence analysis software OMIGA 2.0 (Oxford Molecular Ltd., Oxford, England) was used to design PCR oligonucleotide primer sets for the *fur* gene, to assemble multiple-sequence alignments, and to search for probable ORFs. Statistical analysis of the microarray data was performed using the computer program SAS (SAS Institute, Inc., Cary, N.C.).

RESULTS

Sequence analysis of the *S. oneidensis fur* gene. Annotation of the genome sequence for the metal-reducing bacterium *S. oneidensis* MR-1 (TIGR, unpublished data) revealed the presence of a *fur* homolog (429 bp in size), which was predicted

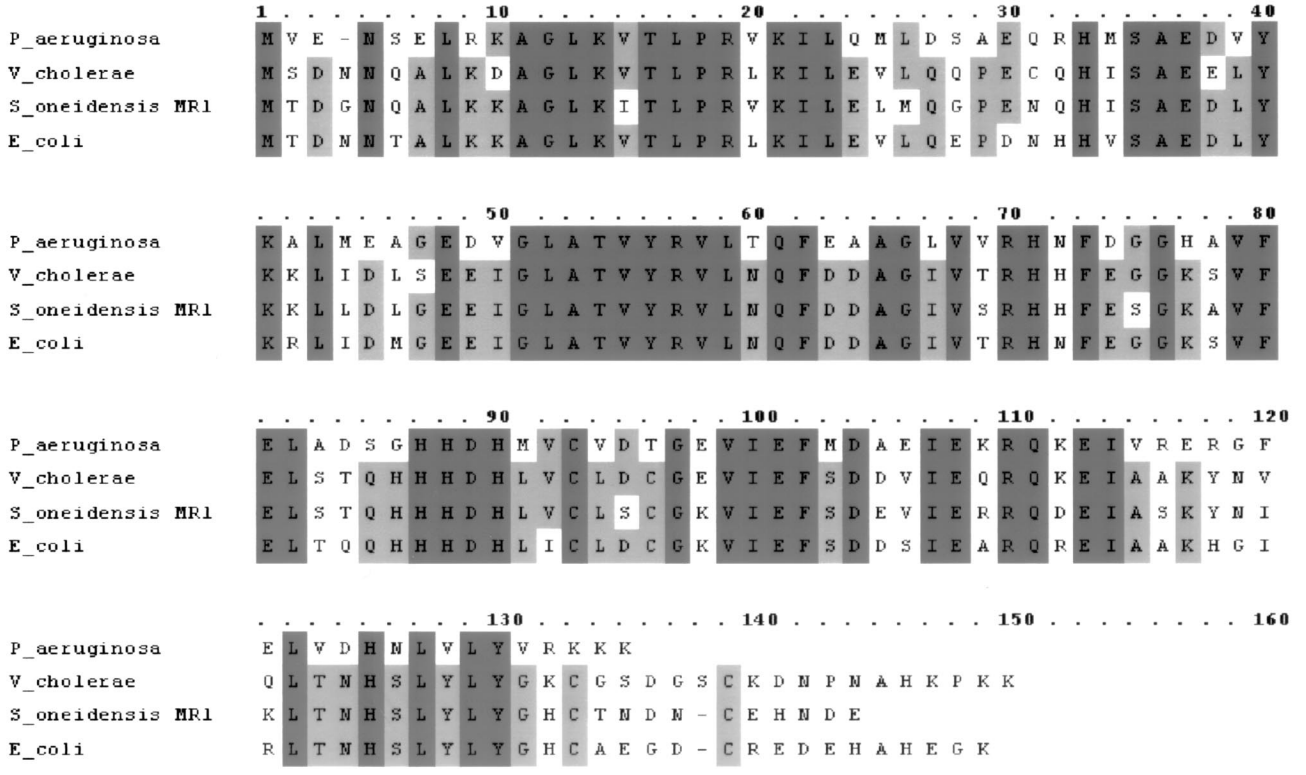

FIG. 1. Amino acid sequence alignment of *fur*-encoded proteins from *P. aeruginosa* (GenBank accession no. A40622), *V. cholerae* (GenBank accession no. A42282), *S. oneidensis* MR-1 (http://www.tigr.org/cgi-bin/BlastSearch/blast.cgi), and *E. coli* (GenBank accession no. S07308). Conserved amino acid residues are shaded.

to be monocistronic based on an analysis of probable ORFs. Comparison of the deduced amino acid sequence showed that *S. oneidensis* MR-1 Fur shares a high degree of identity to its homologs in *E. coli* (73%) and *V. cholerae* (79%) and a lower degree of sequence identity to *P. aeruginosa* Fur (57%) (Fig. 1). This high level of homology at the primary sequence level strongly suggests that these proteins share similar biological functions. The *fur* gene was predicted to encode a 143-amino-acid protein with a predicted molecular mass of 16,286 Da and a pI of 5.57. A potential ribosome-binding site was located 6 nucleotides upstream of the proposed ATG translation start. Analysis of the deduced amino acid sequence of *S. oneidensis* MR-1 Fur also revealed the presence of two potential metal-binding domains rich in histidines and cysteines: a conserved $HHHXHX_2CX_2C$ motif at amino acid residues 86 to 96 and another, less conserved carboxyl-terminal motif (HCX_4CXH) at residues 132 to 140 (Fig. 1).

Transcription of the *E. coli fur* gene is modulated by Fur itself through a single Fur-binding site overlapping the −10 region in the promoter sequence (18). Examination of the 5′ region flanking the *S. oneidensis fur* ORF revealed a putative Fur-binding site that overlapped a consensus −10 promoter element (TATAAT) and was positioned 40 nucleotides upstream of the presumed ATG translational start. The *S. oneidensis* Fur box, TATAATGGCAAGCACTATC, matched the *E. coli* consensus sequence (GATAATGATAATCATTATC) in 14 base positions out of 19 (a 74% identity), suggesting that transcription of *S. oneidensis* MR-1 *fur* may be controlled by an autoregulatory mechanism.

Generation of a *fur* mutant strain. To inactivate the *fur* gene, we utilized the suicide plasmid vector pKNOCK-Km^r, which contained a 179-bp internal fragment of *S. oneidensis* MR-1 *fur*. Disruption of the *fur* gene by suicide plasmid integration was verified by PCR amplification using *fur*-specific primers that flanked the pKNOCK-Km^r insertion sites. As expected, a PCR product approximately 324 bp in size was amplified from wild-type genomic DNA, and a 2.5-kb product, which was consistent with plasmid-interrupted *fur*, was amplified from FUR1 genomic DNA (data not shown). RT-PCR analysis demonstrated that the expected 281-bp RT-PCR product, indicative of *fur* expression, could be detected in wild-type cells but not in the FUR1 mutant strain (data not shown).

Phenotypic characterization of the *fur* mutant. Siderophore biosynthesis by the DSP10 parent and *fur* mutant strains of *S. oneidensis* was compared in LB medium with and without the addition of 50 μM $FeCl_3$ by using the chrome azurol S assay. The *fur* mutant produced approximately threefold more siderophore than the wild type when grown to stationary phase under aerobic conditions. Growth of these strains in the presence of 50 μM $FeCl_3$ reduced siderophore production by the wild-type strain to background levels (at least a 12-fold reduction), whereas siderophore production by the *fur* mutant remained essentially unaffected in response to increased iron levels (data not shown).

To determine whether the *fur* mutation affected anaerobic metabolism, the phenotype of FUR1 was also examined in terms of the ability of the strain to grow on and reduce various electron acceptors under anaerobic respiratory conditions.

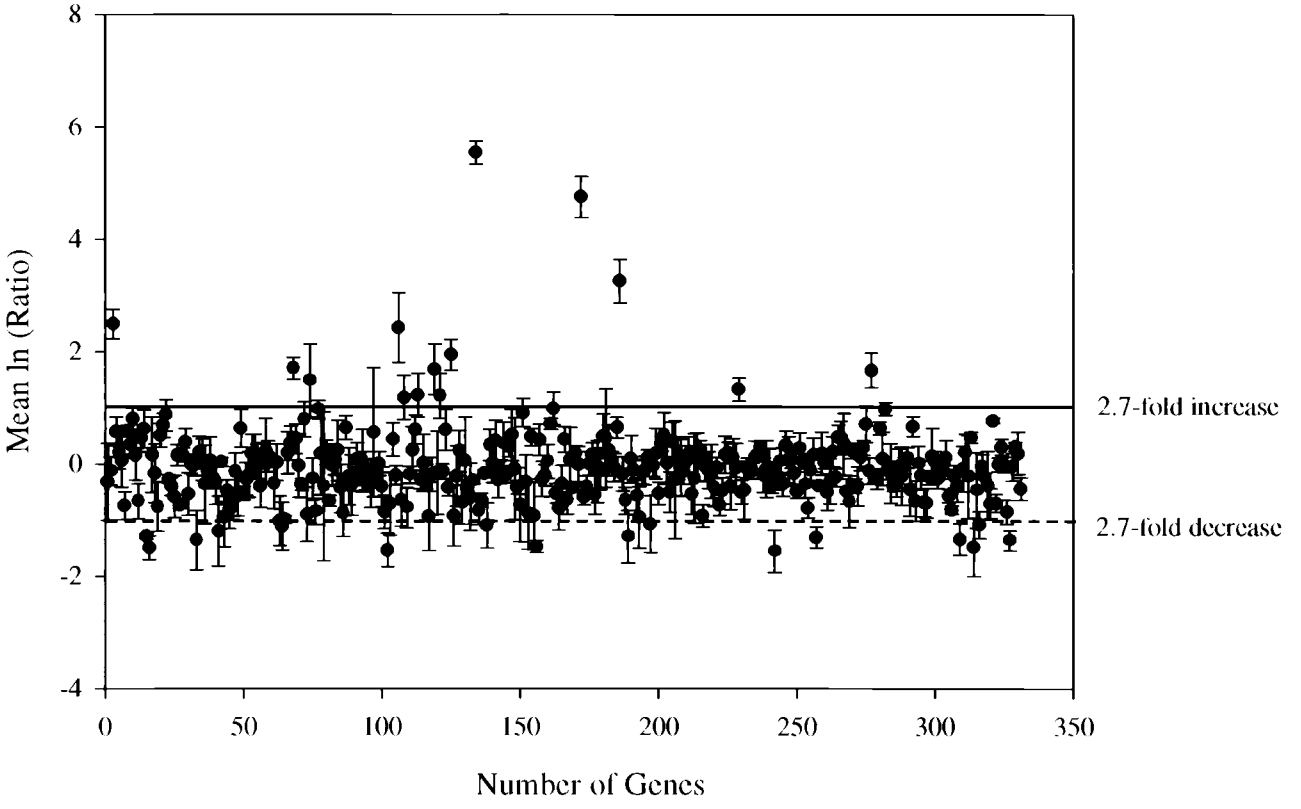

FIG. 2. Analysis of microarray gene expression data. Mean signal intensity ratios of mutant to wild-type mRNA levels were obtained from two to four independent replicate experiments for each gene. Genes with mean fluorescence intensities less than two standard deviations above background in both channels were excluded. Expression ratios were transformed to natural log, such that a 2.7-fold increase (solid line) in transcript levels in the *fur* mutant equaled 1, whereas a 2.7-fold decrease (dashed line) equaled −1. The relative error of each expression ratio is presented.

Based on end point culture turbidity determinations, the *fur* mutant resembled DSP10 in its ability to grow on the following electron acceptors: MnO_2, $Fe(OH)_3$, Fe(III) citrate, nitrate, nitrite, DMSO, TMAO, fumarate, thiosulfate, sulfite, and AQDS (data not shown). In addition, FUR1 retained the ability to reduce Fe(III) and Mn(IV) at rates that were comparable to those of DSP10 (data not shown).

Gene expression profiling of the *fur* mutant. Partial genome microarrays were used to identify genes in *S. oneidensis* MR-1 that were affected by the *fur* mutation. Expression arrays contained 691 different DNA elements, representing approximately 15% of the total protein-coding capacity of the MR-1 genome. These predicted ORFs had annotated functions in energy metabolism, transcriptional regulation, adaptive responses to environmental stress, iron acquisition, substrate transport systems, biosynthesis, and other cellular functions. Gene expression in the *fur* mutant was compared with expression in the wild-type strain under aerobic conditions in the presence of additional iron (50 μM ferric citrate). Experiments in the presence of the iron chelator 2,2′-dipyridyl (0.2 mM) were unsuccessful because of the inability of *S. oneidensis* MR-1 to grow sufficiently under conditions of low iron.

Following microarray data normalization and the removal of low-confidence spot data (36), a population of 331 genes was selected for further analysis. The relative transcriptional responses of each of these genes in the *fur* mutant were expressed as mean natural log-transformed fluorescence ratios and compared (Fig. 2). Of this subset, 30 genes consistently exhibited significant changes (>2-fold differences) in transcription relative to the wild-type control (Table 2). Coefficients of variation were calculated for each differentially expressed gene to determine the total variation in intensity ratios and the reliability of the results. Statistical analysis indicated that the expression levels were significantly different ($0.01 \leq P \leq 0.05$) from the reference for all of the genes listed in Table 2.

Fourteen genes reproducibly displayed a >3-fold-higher transcription level in the *fur* mutant than in the wild type (Table 2). Not surprisingly, eight of these genes grouped within the putative function category of iron acquisition and utilization. Genes coding for a heme-hemopexin utilization protein C (HxuC) and alcaligin siderophore biosynthesis protein (AlcC) showed high-level constitutive expression, with 312-and 137-fold increases, respectively, in mRNA abundance in FUR1. Two different *fhuA* genes (ORFs 1988 and 3509), which encode outer membrane ferrichrome-iron receptor proteins, displayed fold inductions of 29 and 8 in FUR1, while other putative outer membrane iron acquisition genes (*hemR*, *irgA*, *ompW*, and TonB receptor homolog genes) showed 3- to 5-fold increases in transcription.

Other genes with annotated functions in energy metabolism, transcriptional regulation, and oxidative defense also showed altered expression patterns in the *fur* mutant. Interestingly, the

TABLE 2. Summary of gene expression data from microarray analysis[a]

Putative gene function	ORF	Protein, gene	Relative expression (FUR1/WT)[b]		CV[c] (%)	No. of replicates[d]
			Mean	SD		
Iron acquisition and utilization	870	Heme-hemopexin utilization protein C, *hxuC*	312.32	74.45	12.17	16
	207	Alcaligin siderophore biosynthesis protein, *alcC*	137.41	48.48	12.74	15
	1988	Ferrichrome-iron receptor, *fhuA*	29.04	10.13	14.92	16
	3509	Ferrichrome-iron receptor, *fhuA*	8.21	2.31	31.52	14
	3607	Ton-dependent heme receptor A, *hemR*	3.58	0.74	51.56	14
	2722	TonB system receptor	3.15	0.25	35.10	14
	1761	Iron-regulated outer membrane virulence protein, *irgA*	5.64	1.45	17.47	12
	3704	Outer membrane protein w precursor, *ompW*	5.92	3.38	39.82	14
Energy metabolism	562	O_2-independent coproporphyrinogen III oxidase, *yggW*	3.98	0.68	26.50	16
	4405	cbb_3-type cytochrome *c* oxidase, *ccoQ*	0.25	0.07	22.36	13
	4406	cbb_3-type cytochrome *c* oxidase, *ccoN*	0.38	0.07	47.75	11
	4398	Electron transport regulator, *etrA*	0.35	0.08	35.13	13
	2260	Cytochrome *c* maturation protein B	0.36	0.01	11.39	8
	575	Cytochrome b_{561} (putative)	0.24	0.03	16.46	16
	4384	Dihydrolipoamide acetyltransferase, *pdhC*	13.18	2.27	22.88	14
	2336	Citrate synthase 2, *prpC*	15.04	8.32	31.71	14
	2417	Pyruvate dehydrogenase e_1 component, *aceE*	3.20	0.51	39.19	16
	2109	Formate dehydrogenase accessory protein, *fdhD*	2.89	0.20	28.35	16
	4501	Probable quinone oxidoreductase, *qor*	0.30	0.12	33.75	9
	3516	Short-chain alcohol dehydrogenase homolog, *yiaY*	0.26	0.13	34.90	13
	2266	Phosphoenolpyruvate carboxylase, *ppc*	3.66	1.54	42.26	12
Other or unclassified	3920	3-Oxoacid coenzyme A transferase	5.28	1.69	29.37	12
	1824	RNA polymerase sigma-32 factor, *rpoH*	0.45	0.03	25.49	15
	33	SOD (Fe/Mn), *sodB*	0.50	0.09	45.45	8
	4319	16-kDa heat shock protein A, *ibpA*	0.42	0.08	39.27	14
	864	FixG-related protein	0.35	0.10	27.01	14
	4305	NifS protein homolog	0.35	0.15	53.16	15
	3587	Phosphate regulon transcriptional regulator, *phoB*	0.28	0.05	21.72	9
	4233	Transcriptional regulatory protein, *chvI*	0.27	0.05	22.32	14
	484	Cation efflux system protein, AcrB-AcrD-AcrF family	0.23	0.05	20.28	14

[a] Mutant (FUR1) and wild-type strains were grown under aerobic conditions in the presence of 50 μM ferric citrate (iron-replete environment). Total cellular RNAs from these two strains were labeled with either Cy3-dCTP or Cy5-dCTP, and another set of duplicate reactions was carried out with the fluorescent dyes reversed. Sequence annotation was provided as a courtesy of TIGR (unpublished results). ORF designations may change once the *S. oneidensis* MR-1 genome sequence annotation is released.

[b] Relative expression is presented as the ratio of the fluorescence intensity of the *fur* mutant to that of the wild-type (WT) parent strain. Standard deviations for each mean expression ratio are provided.

[c] Coefficient of variation (CV) equals (standard deviation/mean ratio)(100%). The average coefficient of variation for this data set was 29.7%.

[d] Number of replicate data points used in the statistical analysis.

transcription of genes for a number of electron transport-associated components (*ccoNQ*, cytochrome *c* maturation protein B gene, cytochrome b_{561} gene, and *qor*) decreased 2.6- to 4.2-fold in FUR1 grown under aerobic respiratory conditions. A 2.9-fold reduction in expression was observed for *etrA*, a gene encoding a putative electron transport regulator that shares a high degree of primary sequence identity to *E. coli* Fnr (73.6%). Fnr (fumarate-nitrate reduction/regulator) activates transcription of genes encoding anaerobic respiratory functions while repressing expression from some promoters controlling transcription of aerobic respiratory enzymes (29). EtrA appears to play a role in the regulation of anaerobic respiration in *S. oneidensis* (47, 58), but its precise function has yet to be defined.

The expression of other putative regulatory genes declined in the *fur* mutant as well. *rpoH* (encoding the environmental stress [heat shock] sigma-32 factor) (28) was repressed 2.2-fold, while *phoB* (phosphate regulon transcriptional regulator) (70) and *chvI* (virulence regulator) (15), both response regulator-encoding genes in two-component sensory transduction systems, exhibited an approximately 4-fold reduction in mRNA levels (Table 2). Also showing decreased transcription in response to the *fur* mutation were genes encoding homologs for iron/manganese (Fe/Mn)-cofactored superoxide dismutase (SOD) (*sodB* gene) and a cation efflux system protein of the AcrB-AcrD-AcrF family, which exhibited a 4.3-fold reduction in mRNA expression levels. Finally, *pdhC* (dihydrolipoamide acetyltransferase) and *prpC* (citrate synthase 2), genes putatively involved in intermediary carbon metabolism, showed large fold inductions (13.2 and 15, respectively) in expression, while *aceE* (pyruvate dehydrogenase e_1 component) displayed a 3.2-fold increase in transcription in FUR1. A gene coding for an oxygen-independent coproporphyrinogen III oxidase (*yggW*), an enzyme involved in heme biosynthesis under anaerobic conditions, exhibited an approximately fourfold increase in mRNA expression (Table 2).

Analysis of protein expression profiles using 2-D PAGE and mass spectrometry. To investigate alterations in protein expression profiles as a result of the *fur* mutation, we compared 2-D PAGE patterns of whole-cell proteins from *S. oneidensis*

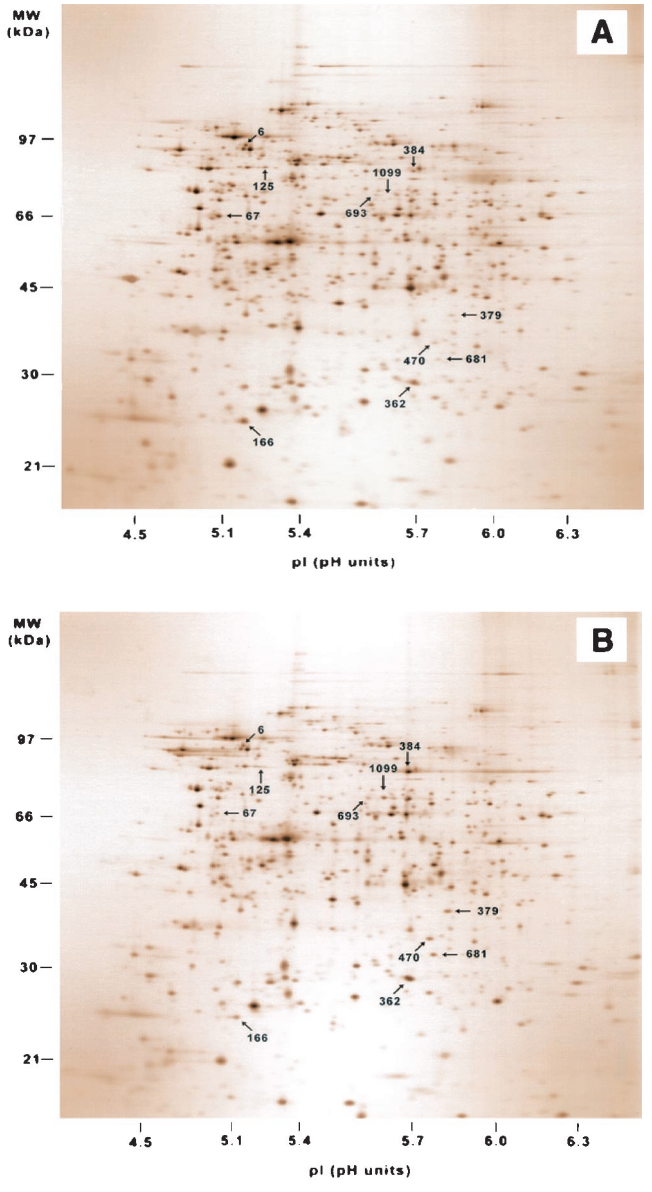

FIG. 3. 2-D PAGE of whole-cell lysates of *S. oneidensis* MR-1 (A) and FUR1 (B) grown in LB medium (high concentration of iron) under aerobic conditions. Protein spots showing significant quantitative differences (a P value of at least <0.05) between the two strains are indicated by arrows and numbers. 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 (MW) standards are presented in kilodaltons along the vertical axis.

MR-1 and FUR1 grown aerobically in LB medium (with a high concentration of iron). Representative 2-D gels of the two strains are presented in Fig. 3. Each of the two samples was subjected to 2-D gel electrophoresis and analyzed in triplicate to enable statistical analysis. Seven major proteins (spots 362, 379, 384, 470, 681, 693, and 1099) consistently detected in both *S. oneidensis* strains were increased significantly ($P < 0.05$) in abundance in mutant cells relative to wild-type cells. A comparison of relative integrated densities averaged from two to four silver-stained gels revealed increases ranging from approximately 2.7- to 11.9-fold relative to the wild-type strain (Table 3). In addition, four protein species (spots 6, 67, 125, and 166) consistently showed decreases in abundance of FUR1 relative to the wild type (Table 3). Spots 67 and 166, in particular, displayed 4.5- and 3-fold decreases, respectively, in protein abundance.

Micro-LC-ESI-MS/MS was used to identify proteins showing significant differences in abundance on 2-D gels. Table 3 presents a summary of the mass spectrometry data, including predicted molecular masses, pI values, and protein identification obtained by using similarity searches based on sequence tags obtained from tryptic peptides. Three of the proteins identified (spots 125, 362, and 470) corresponded to conserved hypothetical proteins of unknown biological function, and the genes encoding these proteins were not included in the microarray experiments. The expression patterns of two other proteins, encoded by genes annotated as Fe/Mn-SOD (spot 166) and AlcC (spot 384), were consistent with their transcript levels as determined by microarray hybridization (Tables 2 and 3). The gene encoding the protein in spot 681, identified by MS/MS as a periplasmic hemin-binding protein (HutB) involved in hemin transport, was not represented on the expression array. Nonetheless, the increased abundance of the predicted HutB in FUR1 is consistent with the role of Fur as a classical transcriptional repressor of iron transport genes. Other proteins that exhibited perturbations in abundance were identified as translation elongation factor G, agglutination protein, cysteine synthase A, prismane (protein with Fe-S clusters), and a putative phosphomannomutase (Table 3). Of these proteins, only the agglutination protein-encoding gene was not represented on the microarrays. Although phosphomannomutase showed the largest increase (11.9-fold) in protein abundance, a corresponding increase in mRNA levels for the gene was not observed in the microarray analysis.

TABLE 3. Summary of MS/MS data for protein spots showing altered expression levels on 2-D gels for wild-type and *fur* mutant cell extracts

Spot no.	Mol wt	pI	Protein identity	Abundance (FUR1/WT ratio)[a]
6	76,950	5.04	Translation elongation factor G	0.66
67	52,343	5.17	Agglutination protein	0.22
125	65,642	5.13	Conserved hypothetical protein	0.61
166	21,495	5.20	SOD (Fe/Mn), SodB	0.35
362	30,777	6.46	Conserved hypothetical protein	3.61
379	36,092	6.93	Cysteine synthase A	2.72
384	72,053	5.60	AlcC protein	5.19
470	30,233	6.93	Conserved hypothetical protein	2.93
681	33,746	6.60	Periplasmic hemin-binding protein, HutB	4.39
693	60,502	5.41	Prismane	2.81
1099	62,212	5.64	Phosphomannomutase, putative	11.89

[a] 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 in FUR1 to that in the wild-type (WT) strain. Statistical analysis of the relative protein abundance was accomplished using a two-tailed Student t test. Only those proteins displaying quantitative differences with a P value of at least <0.05 were considered to differ significantly in abundance between the mutant and wild-type samples.

DISCUSSION

With the availability of whole-genome sequences, the next challenge is to empirically confirm the annotated functions and to provide biological meaning to genes assigned unknown functions by using integrative experimental approaches. The value of DNA expression microarrays for the analysis of genetic mutants is clear from previous studies (see, for example, references 45 and 65). In this study, microarray-based transcription profiling, physiological assays, and proteomic tools were used to investigate the effect of an insertional null mutation of the *fur* gene on expression patterns in the dissimilatory metal-reducing bacterium *S. oneidensis* MR-1.

The results described here agree with the well-established model of Fur as a negative regulator of siderophore/receptor-mediated iron acquisition and further implicate *S. oneidensis* MR-1 Fur in previously unidentified functions. DNA microarray analysis revealed that disruption of the *Shewanella fur* locus resulted in constitutive expression of genes (*hxuC*, *alcC*, *fhuA*, *hemR*, *irgA*, *ompW*, and a TonB receptor homolog gene) with annotated functions in iron transport and assimilation (Table 2). The transcription of most of these same genes (specifically, *hxuC*, *alcC*, *hemR*, *irgA*, *ompW*, and both *fhuA* genes) in wild-type *S. oneidensis* MR-1 was repressed during anaerobic respiratory growth in the presence of fumarate, Fe(III), or nitrate as the terminal electron acceptor, with the greatest fold reductions observed under Fe(III)-reducing conditions (8). The loss of iron repression of siderophore expression in the mutant strain, FUR1, as measured by the chrome azurol S assay, supported the microarray data and was consistent with phenotypes reported for other *fur* mutants (10). The two most highly derepressed genes were a predicted *hxuC* gene, which encodes a heme-hemopexin utilization protein C exhibiting 31% sequence identity to *Haemophilus influenzae* (type b) *hxuC* (GenBank accession no. U09840), and *alcC*, which shares 48% amino acid sequence identity to its homolog (GenBank accession no. U61153) in the *Bordetella bronchiseptica alcABC* operon. The *H. influenzae hxuCBA* operon is required for the utilization of free heme and heme bound to the human serum protein hemopexin (16, 30), whereas the *alc* gene cluster in *Bordetella* species is involved in the biosynthesis of the macrocyclic dihydroxamate siderophore alcaligin and is under the control of Fur (6, 10, 38, 39). Multiple putative Fur-binding sites were identified in the upstream regions flanking *hxuC* and the *alc* gene cluster in *S. oneidensis* MR-1 (Table 4). These potential Fur boxes exhibited 47 to 63% sequence identity, corresponding to matches of 9 out of 19 to 12 out of 19 to the *E. coli* Fur box consensus sequence. Further studies are required to determine whether these sequence elements represent functional targets for Fur-specific binding.

Genes encoding outer membrane receptor proteins (FhuA, HemR, IrgA, and TonB system receptor) also exhibited derepression in the *fur* mutant. The *S. oneidensis irgA* homolog shares 51% amino acid sequence identity to the *V. cholerae* virulence-associated gene *irgA* (26). Fur has been shown to control the expression of several virulence determinants in known microbial pathogens (13, 14), including *irgA* from *V. cholerae* (25, 71). It is important to note, however, that IrgA from *V. cholerae* is most closely related to iron-regulated ferric siderophore receptors (25) and therefore its likely role in virulence is in iron acquisition. Scrutiny of the promoter region for the *Shewanella irgA* homolog revealed two overlapping potential Fur boxes showing 47 and 68% identity to the *E. coli* consensus sequence (Table 4). Because of its wide distribution in nature, it is conceivable that *S. oneidensis* MR-1 would utilize a number of different iron transport mechanisms for its establishment in various environmental niches.

Although phenotype studies indicated that the *S. oneidensis fur* mutant was not impaired in growth or in the utilization of various electron acceptors under anaerobic conditions, genes involved in electron transport systems (cbb_3-type cytochrome *c* oxidases, cytochrome *c* maturation protein B, cytochrome b_{561}, and a probable quinone oxidoreductase) and the putative electron transport regulator-encoding gene, *etrA*, displayed decreased transcription in the FUR1 strain under aerobic respiratory conditions (Table 2). Fur box-like elements were also identified in the promoter regions for all of these genes (Table 4), with putative Fur-binding sites upstream of genes for cytochrome *c* maturation protein B and cytochrome b_{561} showing the highest sequence identity (53 to 68%) to the consensus. Interestingly, the upstream region of *etrA* contained an Fnr box-like sequence (TTGAT-N_4-cTCgc) that displayed 70% identity to the consensus Fnr-binding site sequence (TTGAT-N_4-ATCAA) (29) and overlapped a Fur box-like element with 10 matches of 19 to the consensus. The *S. oneidensis etrA* gene, which shows striking sequence identity to *E. coli* Fnr at the amino acid level, contains the four conserved cysteine residues of *E. coli* Fnr and the C-terminal helix-turn-helix motif that are required for iron-sulfur coordination and DNA-binding activity, respectively (58).

Despite the observation that *Shewanella* EtrA can complement an *fnr* mutant of *E. coli* (58), the biological role of EtrA has not been definitively resolved. Recently, Maier and Myers (47) showed that while an *etrA* knockout strain (ETRA-153) was able to grow on and/or reduce various electron acceptors, ETRA-153 had reduced initial growth rates on fumarate and nitrate, which correlated with lower fumarate and nitrate reductase activities. This work suggested that EtrA might play a subtle role in MR-1 anaerobic gene regulation (47). In the study described here, microarray analysis indicated that *etrA* transcript abundance was approximately threefold lower in aerobically grown FUR1 than in the wild type (Table 2). To determine whether the *fur* mutation affected initial growth rates on fumarate and nitrate, short-term anaerobic growth on these electron acceptors was examined in LB medium at 30-min intervals. In contrast to the case for the *etrA* mutant ETRA-153 (47), the initial growth rate of FUR1 on fumarate was comparable to that of MR-1 (Fig. 4A). The growth rates of MR-1 and FUR1 on nitrate were nearly identical over the first hour, after which the growth rate of FUR1 was approximately 62% lower than that of MR-1 (Fig. 4B). At this point, we cannot explain the difference in growth rates for FUR1 on fumarate and nitrate. Nonetheless, the microarray data and sequence analysis suggest that Fur may act with EtrA and possibly other regulatory proteins to coordinate the synthesis of iron-containing enzymes and cytochromes with iron uptake and respiration. Previous research has demonstrated that expression of *E. coli sodA*, the gene encoding Mn-cofactored SOD, is coordinately regulated by the global control systems of Fur, Arc, and Fnr (32). Further studies with strains harboring

TABLE 4. Predicted Fur-binding sites located upstream of genes showing altered expression in FUR1 mutant

Gene, protein (ORF)	Putative Fur box element[a]	Match out of 19-mer consensus	Distance from translational start[b]
hutA, heme-hemopexin utilization protein C (870)	GATAATGATttctATTtgaatTgATtAatAaaATTAat	12	165
		9	36
alcC, alcaligin siderophore biosynthesis (207)	GATAATatTtATataaATaaATAtTtATAtaaATacTttATA ATttTtAagATgcTa	11	337
		10	334
		10	188
fhuA, ferrichrome-iron receptor (1988)	aAcAATtcTtATttaTATt	10	52
fhuA, ferrichrome-iron receptor (3509)	aATAATtATtAatcaTATaaATcATtATcATtcgcAaC	11	392
		10	42
hemR, Ton-dependent heme receptor A (3607)	GATtcTtATggTttTTtTCGAgAATtATtcTCATctTtaATtA TtcTcATCtTTggC	11	138
		12	78
		11	75
TonB system receptor (2722)	aAcAATtcTcATttcTAag	9	33
irgA, iron-regulated outer membrane virulence protein (1761)	GAaAATtATtATCATTtagaATtATtATcATttagAaa	13	46
		9	43
ompW, outer membrane protein w precursor (3704)	aATAAgtAgcAcCAcTtaa	9	103
yggW, O_2-independent coproporphyrinogen III oxidase (562)	GATAAacATcgcCcaatga	8	31
ccoQN, cbb$_3$-type cytochrome *c* oxidase (4405–4406)	agTAATaAgcAgtggaAgCaATAAgcAgtggaAgcATt	8	22
		8	19
etrA, electron transport regulator (4398)	GATtATGcTcgcCtTTggtacTAtTaATcgTCtTTggt	10	227
		9	113
Cytochrome *c* maturation protein B (2260)	aATAAatcTcATCtTTtTgGAgAAatATtgcggTTATt	11	236
		10	127
Cytochrome b_{561} (putative)	GATAATGAacATtATTtaaGAacATtATTtaatTaATC	13	56
		10	49
pdhC, dihydrolipoamide acetyltransferase (4384)	aATgtTcATccggcaTATt	8	76
prpC, citrate synthase 2	GATAtTGAgccTgtcTgcaaATAAccATAgcgtTaAcg	9	50
		9	32
aceE, pyruvate dehydrogenase e_1 component (2417)	cATAAaaATtggtAagAcC	9	44
fdhD, formate dehydrogenase accessory protein (2109)	accAATtgTttTtATTtgaaATtgTttTtATttgaATt	8	42
		8	39
qor, probable quinone oxidoreductase (4501)	aATAATGAcctagcTTAggGtTAAaaATtgTgcTTtgt	10	95
		9	35
yiaY, short-chain alcohol dehydrogenase homolog (3516)	GtTcATtATttagtTatct	7	153
ppc, phosphoenolpyruvate carboxylase (2266)	GATttTtATgtTttTctat	8	30
3-Oxoacid coenzyme A transferase (3920)	GATAAaGATcATggcatTa	11	10
rpoH, RNA polymerase sigma-32 factor (1824)	GATAAgtAgcAagAgagTa	9	28
sodB, SOD (Fe/Mn) (33)	aAcAATGATtcaCATcAcaaATgATtcacATCAcaAat	11	91
		9	88
ibpA, 16-kDa heat shock protein (4319)	GcTAATttcccatATTgct	8	31
FixG-related protein (864)	atTAAatATcAatATatTa	9	89
NifS protein homolog (4305)	GATAAaaActATttTTgTa	11	64
phoB, phosphate regulon transcriptional regulator (3587)	GAcAATtATcgcgAcTATaaATAATagTcgTgtTgAca	11	105
		9	40
chvI, transcriptional regulatory protein (4233)	aATAAattcctgtATagTC	8	59
Cation efflux system protein (484)	tAaAATtAaccTaAcgcTt	8	36
Consensus	GATAATGATAATCATTATC	19	

[a] Nucleotides matching those of the *E. coli* consensus Fur box element are shown in uppercase letters.
[b] The position of the putative Fur box is given as the distance from the 5′ end of the motif to the presumed translation start codon.

mutations in multiple regulatory genes are needed to confirm whether coordinate regulation of energy metabolism occurs in *S. oneidensis* MR-1.

Mutations in *fur* have been reported to have pleiotropic effects in other species (21, 43, 63, 67). Similarly, the *fur* mutation in *S. oneidensis* appeared to have a broad effect on gene expression profiles. Altered expression levels were also observed for putative transcriptional regulator genes (*rpoH*, *phoB*, and *chvI*), the oxidative stress protective gene *sodB*, and genes involved in energy/intermediary carbon metabolism (*pdhC*, *aceE*, *prpC*, *ppc*, *fdhD*, and *yiaY*), as well as other genes encoding a cation efflux system protein, 3-oxoacid coenzyme A transferase, FixG-related protein, and a NifS protein homolog (Table 2). Putative Fur box elements with weak sequence identities (37 to 58%) to the consensus were identified in the upstream regions of all of these genes (Table 4). Like that of the electron transport-associated genes, transcription of the response regulatory genes *rpoH*, *phoB*, and *chvI* decreased in FUR1, suggesting that expression of these genes may be under some form of Fur-mediated positive control. To our knowledge, *rpoH*, *phoB*, and *chvI* have not been shown to be members of the Fur regulon in other bacteria. Reduced transcript and protein expression levels for SodB (Tables 2 and 3) also suggest that positive regulation by Fur might be operative in *S. oneidensis* MR-1. It is important to note that *E. coli* Fe-SOD is positively regulated by Fur (51),

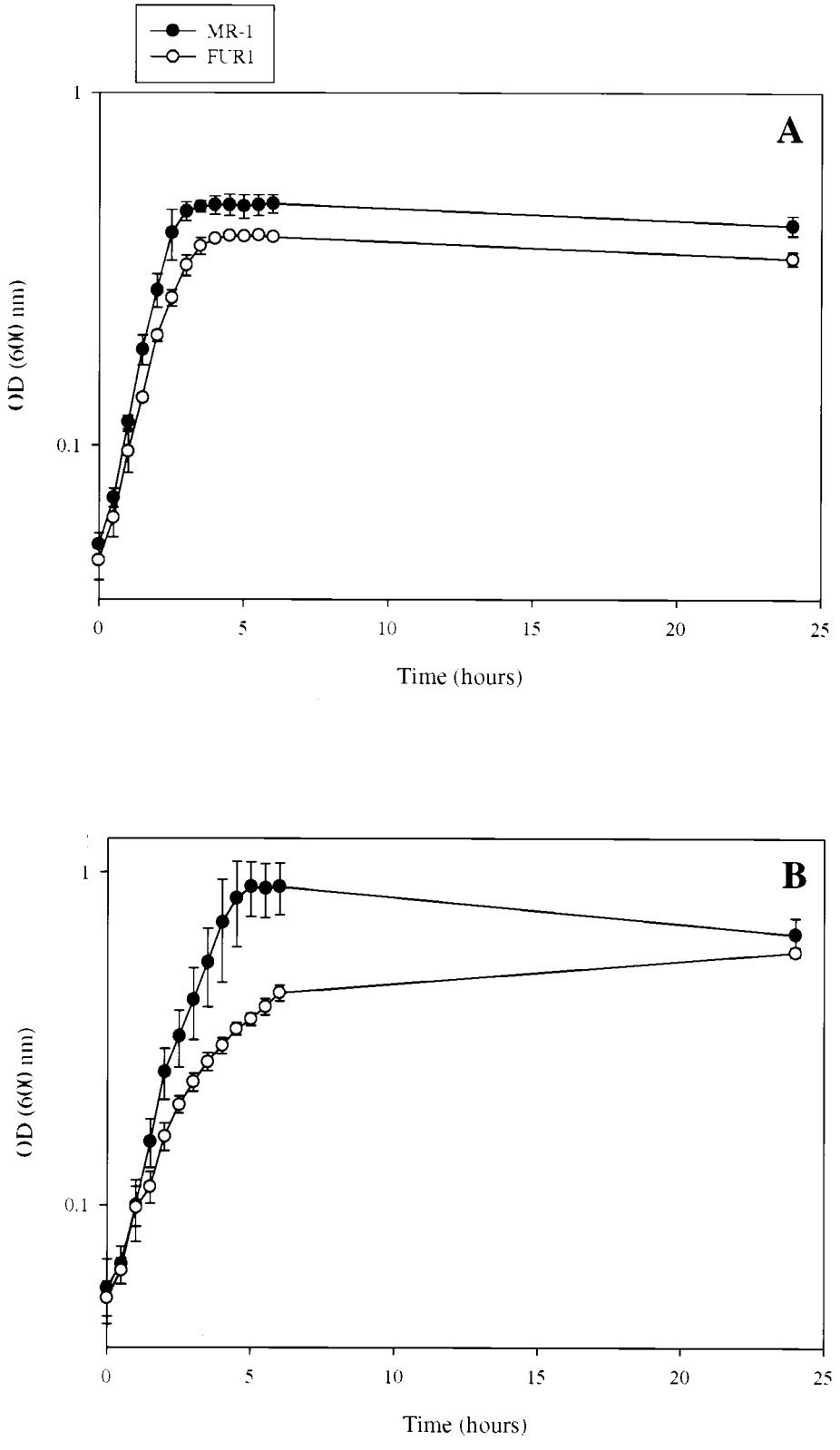

FIG. 4. Anaerobic growth of MR-1 and FUR1 strains of *S. oneidensis* on LB medium supplemented with 20 mM Na lactate and 10 mM fumarate (A) or 8 mM nitrate (B). Cultures pregrown anaerobically overnight on the tested substrates were used to inoculate the medium. Values represent independent means ± standard deviations of OD_{600} readings obtained for triplicate cultures of each strain assessed in parallel.

and decreased Fe-SOD activity was observed in a *fur* mutant of *P. aeruginosa* (34). Finally, expression levels for *prpC*, *ppc*, *fdhD*, and genes encoding enzyme components of the pyruvate dehydrogenase complex (*pdhC* and *aceE*) were elevated in the *S. oneidensis fur* mutant, suggesting a regulatory role of Fur in the tricarboxylic acid cycle and other pathways for energy metabolism. No other report, to our knowledge, has demonstrated the effect of a *fur* mutation on these specific energy metabolism genes, although *P. aeruginosa* Fur has been shown to regulate fumarase in the tricarboxylic acid cycle (33) and a subunit of complex I (NADH:ubiquinone reductase) of the electron transport chain (53).

The effect of a *fur* mutation in *S. oneidensis* MR-1 was explored further through the use of 2-D gel electrophoresis and micro-LC-ESI-MS/MS to compare protein expression profiles in wild-type *S. oneidensis* and the *fur* mutant. Although some putative membrane-associated proteins (spots 67/ORF01553 and 125/ORF03403 in Table 3) were identified, membrane proteins were not specifically analyzed. Proteomic analysis indicated that the Fur regulon in *Shewanella* appears to be complex, affecting other cellular processes besides iron acquisition systems. In addition to the expected members of the Fur regulon (e.g., AlcC, HutB, and SodB), three conserved hypothetical proteins, translation elongation factor G, an agglutination protein, cysteine synthase A, prismane, and phosphomannomutase showed altered abundance levels in the *fur* mutant compared to the wild type. Sequence analysis indicated that the gene encoding hypothetical protein 362 clustered with genes involved in a putative sulfate transport system. Prismane is a hybrid iron-sulfur cluster protein (55, 64) and may play a role in aerobic-anaerobic respiration, although its function has not been clearly elucidated. We did not observe measurable differences in protein abundance for other genes showing differential expression on microarrays. However, it is important to note that identification of proteins was limited to those species that fell within a certain molecular mass and narrow pI range (pH 4 to 7) and were of sufficient abundance to be accurately detected and resolved in our 2-D PAGE system.

The microarray-based transcriptional profiling and proteomic analyses presented in this paper provide evidence that Fur functions as a negative regulator of siderophore production and of other genes encoding iron acquisition capabilities in *S. oneidensis* MR-1. Although this study is not a full description of the Fur regulon, the findings also suggest that Fur is a global regulator that appears to positively control genes involved in electron transport systems, the cellular defense against oxygen toxicity, and the regulation of certain adaptive stress responses. More research is needed to establish whether communication between Fur and other regulators such as the Fnr-like EtrA is required for the intricate coordination between intracellular iron levels and the synthesis of iron-containing proteins involved in respiration and oxygen radical detoxification.

ACKNOWLEDGMENTS

We thank Guangshan Li for PCR amplification of *S. oneidensis* MR-1 ORFs, John Heidelberg for *S. oneidensis* MR-1 genome sequence information, and Allison Murray for advice on microarray data analysis.

This research was supported by the U.S. Department of Energy under the Microbial Genome Program and the Natural and Accelerated Bioremediation Research Program of the Office of Biological and Environmental Research. The 2-D PAGE aspect of this work was done at Argonne National Laboratory under U.S. Department of Energy contract no. W-31–109-ENG-38. Oak Ridge National Laboratory is managed by the University of Tennessee-Battelle LLC for the Department of Energy under contract DE-AC05-00OR22725.

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