Analysis of a ferric uptake regulator (Fur) mutant of *Desulfovibrio vulgaris* Hildenborough

Kelly S. Bender[1, †], Huei-Che Bill Yen[1,], Christopher L. Hemme[3,4], Zamin Yang[3], Zhili He[3,4], Qiang He[5,] Jizhong Zhou[3,4], Katherine, H. Huang[6], Eric J. Alm[6,7], Terry C. Hazen[8], Adam P. Arkin[6,9,10], and Judy D. Wall[1*,‡]

[1]Department of Biochemistry, University of Missouri-Columbia, Columbia, Missouri 65211, [3]Environmental Sciences Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831, [4]Institute for Environmental Genomics, Department of Botany and Microbiology, University of Oklahoma, Norman, Oklahoma 73019, [5]Department of Civil and Environmental Engineering, Temple University, Philadelphia, PA 19122 [6]Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, California 94720, [7]Biological Engineering Division, Massachusetts Institute of Technology, Cambridge, MA 02139 [8]Earth Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, California 94720, [9]Department of Bioengineering, University of California, Berkeley, California 94720, [10]Howard Hughes Medical Institute, Chevy Chase, Maryland 20815.

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[†]Present address: Department of Microbiology, Southern Illinois University, Carbondale, Illinois 62901.

*Corresponding author. Mailing address: Department of Biochemistry, 117 Schweitzer Hall, Columbia, MO 65211. Phone: (573) 882-8726. Fax: (573) 882-5635. Email: wallj@missouri.edu.

‡All authors are affiliated with the Virtual Institute for Microbial Stress and Survival (http://vimss.lbl.gov).

ABSTRACT

Previous experiments examining the transcriptional profile of the anaerobe *Desulfovibrio vulgaris* have demonstrated up-regulation of the Fur regulon in response to various environmental stressors. To test the involvement of Fur in the growth response and transcriptional regulation of *D. vulgaris*, a targeted mutagenesis procedure was used for deleting the *fur* gene. Growth of the resulting Δ*fur* mutant (JW707) was not affected by iron availability, but did exhibit increased sensitivity to nitrite and osmotic stresses when compared to the wild type. Transcriptional profiling of JW707 indicated that iron-bound Fur acts as a traditional repressor for ferrous iron uptake genes (*feoAB*) and other genes containing a predicted Fur binding site within their promoter. Despite the apparent lack of siderophore biosynthesis genes within the *D. vulgaris* genome, a large 12 gene operon encoding orthologs to TonB and TolQR also appeared to be repressed by iron-bound Fur. While other genes predicted to be involved in iron homeostasis were unaffected by the presence or absence of Fur, alternative expression patterns that could be interpreted as repression or activation by iron-free Fur were observed. Both the

physiological and transcriptional data implicate a global regulatory role for Fur in the sulfate-reducing bacterium *D. vulgaris*.

INTRODUCTION

Iron is an essential nutrient for most bacteria because of its role as an enzymatic co-factor and electron transport protein component. In addition to the metabolic importance of iron, pathogenic bacteria use its availability as an environmental signal for regulation of virulence genes. Despite the metabolic dependence on iron, cellular concentrations must be intricately regulated in aerobic environments to prevent Fe(II)-mediated formation of reactive oxygen species via Fenton chemistry (72). In most bacteria, this complex regulation is carried out by the ferric uptake regulator (Fur) protein (23, 29). The traditional mode of Fur regulation has been described as follows: under iron-replete conditions, the Fur protein and its co-repressor (Fe(II)) block the transcription of iron uptake and storage genes. When iron becomes limiting, the Fur repressor is no longer saturated with Fe(II) and cannot bind the operator, leading to transcription of genes involved in iron uptake and storage. Since iron is found predominantly as insoluble ferric hydroxides in aerobic environments, bacteria have evolved a mechanism for uptake dependent on the synthesis and transport of specialized chelators called siderophores. Thus Fur also regulates the synthesis of siderophores in bacteria studied to date (29, 65, 68). In addition to its role as the primary regulator responding to available iron, Fur has also been shown to play a global regulatory role in oxidative stress response, acid tolerance response, virulence factor synthesis, and motility (2, 23, 29).

Because of the insolubility and sequestration of iron in aerobic and in host environments, studies involving iron regulation have focused primarily on aerobic/facultative and pathogenic microbes, respectively. The role of Fur or iron metabolism in strict anaerobes has received little attention. In anaerobic environments, enough iron is expected to be in the Fe(II) form that its accessibility should not be limiting. Also the likelihood of Fe(II)-mediated formation of reactive oxygen species via Fenton chemistry is decreased by limited oxygen exposure. Thus elaborate iron regulation would seem to be less critical in anaerobes. However, recent genomic studies in metal-reducing δ-proteobacteria have indicated the presence of not one, but three *fur* paralogs, *fur* (DVU0942), *perR* (DVU3095), and *zur* (DVU1340), in these anaerobes (61). While the roles of these regulators remain unclear, the sulfate reducers *Desulfovibrio vulgaris* Hildenborough and *Desulfovibrio desulfuricans* G20 appear to have extended Fur regulons compared to the predictions from genomes of other δ-proteobacteria (61). Regulon members include ferrous iron transporter genes (*feoAB,* DVU2572/71), a flavodoxin (*fld,* DVU2680), P-type and ABC ATPases, and genes possessing GGDEF- and HD-domains. The proteins encoded in the latter genes are predicted to have cyclic di-GMP synthesis and hydrolysis activity, respectively, that could allow second messenger concentrations to be responsive to Fur signals (66). A large cluster of genes predicted to be involved in biopolymer transport, such as *tonB* (DVU2390), were also suggested to be part of the Fur regulon (61). However, siderophore production by *Desulfovibrio* has not been documented nor have genes for siderophore synthesis been identified. The annotation of a putative transporter for the siderophore enterobactin

(*fepC*, DVU0648) suggests a possible mechanism for acquiring insoluble iron via chelators produced by other bacteria.

Desulfovibrio species are anaerobic sulfate-reducing bacteria (SRB) known for their ability to corrode ferrous metals as well as to reduce heavy metals such as uranium (VI), chromium (VI), and technetium (VII). *D. vulgaris* Hildenborough is also believed to possess a robust iron requirement based on its abundance of iron-containing cytochromes, hydrogenases, and electron transport proteins, as determined from genome analysis (36). Despite the solubility of iron in anaerobic environments, the degree to which Fe(II) is accessible to sulfate reducers is unknown, especially since sulfide, a by-product of sulfate reduction, complexes with Fe(II) to form insoluble pyrite (FeS). Another notable aspect of iron metabolism in *Desulfovibrio* is the production of a ferritin protein containing a unique heme group (58, 62). Ferritin and bacterioferritin proteins are produced by aerobic bacteria to sequester free iron in a non-reactive insoluble Fe(III) form (1, 2, 72). The role of these proteins in anaerobic bacteria has been assumed to be in oxygen defense (60, 62), with their intrinsic regulatory mechanisms currently unknown.

Even though *Desulfovibrio* are considered strict anaerobes, they do possess numerous oxygen detoxification proteins such as rubrerythrin, rubredoxin oxidoreductase, and superoxide dismutase, all of which contain iron (19, 24, 45). The role of iron in response to oxygen stress was underscored by recent research with *Bacillus subtilis* indicating that iron oxidation was likely the initiating event for detection of peroxides via the metal-dependent peroxide sensor/regulator PerR (41). The annotation of a *perR* gene in SRB suggests that the relationship between these anaerobes and iron

may be more complex than previously considered. Here we describe a marker exchange method for gene deletion in *D. vulgaris* Hildenborough and the physiology and transcriptional profile of the resulting Δ*fur* strain. The transcriptional data are strengthened by comparison with previous studies documenting the differentially expressed genes in stressed *D. vulgaris* cells (12, 35, 46, 47). While Fur in *D. vulgaris* appears to regulate genes traditionally involved in Fe(II) uptake, our data suggest a diverse regulatory pattern for Fur in sulfate-reducing bacteria. To our knowledge this is the first report of a Δ*fur* deletion in a sulfate-reducing bacterium.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains and vectors used in this study are listed in Table 1. *D. vulgaris* cultures were grown anaerobically as previously described (57) in two defined media, LS4D, routinely used in transcriptomic and proteomic studies of this bacterium so data can be compared, and Yen45, used to reduced the formation of precipitates when ion concentrations were altered. LS4D (60 μM total iron) consisted of: 60 mM sodium lactate, 50 mM Na_2SO_4, 8.0 mM $MgCl_2$, 20 mM NH_4Cl, 2.2 mM K_2HPO_4, 0.6 mM $CaCl_2$, 30 mM PIPES, 12.5 ml trace mineral solution per liter (7), NaOH to a pH of 7.2, and 1.0 ml 10X vitamin solution per liter (7) (added after autoclaving). Yen45 (30 μM total iron) consisted of: 60 mM sodium lactate, 30 mM Na_2SO_4, 8.0 mM $MgCl_2$, 20 mM NH_4Cl, 2 mM phosphate buffer (K_2HPO_4/NaH_2PO_4, pH 7.4), 0.6 mM $CaCl_2$, 30 mM Tris-HCl (pH 7.4), and, per liter, 2 ml modified trace mineral solution (7) (modified by omission of nitrilotriacetic acid and $FeCl_2$), 0.24 ml iron solution (125 mM $FeCl_2$/250 mM EDTA, pH 7.3), 20 ml sterile 1M

$NaHCO_3$ (added after autoclaving), and 1.0 ml sterile 10X vitamin solution (7) (added after autoclaving). As the reductant for both media, 5 ml per liter of an anaerobic titanium citrate solution was used. This solution contained 20% (wt/vol) titanium(III) chloride, 0.2 M sodium citrate, and 8.0% (wt/vol) sodium carbonate. For plating, LS4D medium was supplemented with 0.2% (wt/vol) yeast extract, 1.5 % (wt/vol) agar, and 1.2 mM thioglycolate. Cells were distributed in 4 ml of molten top agar (30 mM PIPES, 1.5% agar).

Deletion cassette construction. Deletion cassettes were constructed similar to molecular bar-coding methods described for yeast (27, 69). Briefly, PCR primer sets were designed to amplify approximately 800 bp up- and downstream of the *fur* ORF (DVU0942). Primer nucleotide sequences are listed in Table 2 along with genome location relative to the *fur* ORF. For future tracking of the mutant in a mixed population, unique barcode sequences were added between the common sequences and Km^R sequences of primers P5 and P6. The following PCR reaction mixture was used to amplify the upstream (primers P1/P2) and downstream (primers P3/P4) regions flanking the *fur* ORF from *D. vulgaris* as well as the Km^R cassette from pSC27 (63) (primers P5/P6): 1X Herculase® buffer (Stratagene, La Jolla, CA), 0.25 mM each dNTP, 2.5 pmol each primer, 2.5 units Herculase® polymerase (Stratagene), and 1 µl template DNA in a total volume of 40 µl. Reactions were cycled according to the following program: 94ºC denaturation for 60 sec; followed by 5 cycles consisting of 94ºC for 30 sec, 45ºC for 30 sec, and 70ºC for 70 sec; then 24 cycles consisting of 94ºC for 30 sec, 54ºC for 30 sec, and 70ºC for 70 sec; and ending with a 6-min extension at 70ºC. Products were gel

purified using Quantum Prep® Freeze'N Squeeze™ DNA Gel Extraction Spin Columns (Bio-Rad, Hercules, CA) and immediately used for fusion PCR.

For the fusion PCR the following reaction mixture was used: 1X Herculase buffer, 0.25 mM each dNTP, 4 units Herculase polymerase, and 50-100 ng of each of the three PCR products generated above (approximately 3 µl each). Before adding the polymerase, the reaction was heated to 94ºC for 30 sec. Reactions were cycled according to the following program: 4 cycles consisting of 94ºC for 30 sec, 55ºC for 60 sec, 72ºC for 3 min 30 sec; then addition of 4 U more of polymerase and 25 pmol each of primers P1 and P4. This mixture was then cycled 25 times at 94ºC for 30 sec, 55ºC for 60 sec, and 72ºC for 3 min 30 sec; and ending with a 8-min extension at 72ºC for 8 min. The resulting fusion PCR product was gel purified as described above and ligated into the *Eco*RV site of pBluescript® (SK+) (Stratagene, La Jolla, CA) to generate pMO707. Prior to electroporation of pMO707 into *D. vulgaris*, the construct was sequenced to check for proper arrangement and lack of sequence errors.

Transformation and mutant selection. Approximately 10^9-10^{10} *D. vulgaris* cells, harvested at early stationary phase ($O.D._{600}$ of ca. 1.0) from LS4D modified to contain 0.2 % yeast extract, were electroporated with 5 µg of the knockout vector (pMO707) and 1 µg lambda DNA, as additional substrate for nucleases in the recipient. Prior to electroporation, the cells were washed two times with ice-cold 1 mM $MgCl_2$/10% (vol/vol) glycerol. Electroporations were carried out in a total volume of 75 µl with a BTX electroporation pulse generator, model ECM630 (Genetronix, San Jose, CA). The parameters obtained for the electroporations were: 1.75 KV, 25 µFD, and 250 ohms. Immediately following transformation the cells were recovered in 1 ml LS4D medium

supplemented with 0.2% (wt/vol) yeast extract. Following four hours of incubation at 37°C, the cultures were diluted to 5 ml in the same medium containing 400 µg G418/ml and allowed to grow overnight. The next day the transformations were plated (1 ml/plate) on solidified LS4D plus 0.2% (wt/vol) yeast extract medium containing 400 µg G418/ml. Resulting transformants were analyzed for deletion of the *fur* gene via PCR that targeted genome regions outside of the *fur* knockout cassette (primers P7 and P8 (Table 2)) and Southern analyses of genomic DNAs. One confirmed *fur* deletion mutant was selected and designated JW707.

Nucleic acid procedures. Genomic DNA was extracted using Wizard® Genomic Purification kit (Promega, Madison, WI). Southern analyses employed Zeta-Probe® (Bio-Rad, Hercules, CA) membrane and were performed according to the manufacturer's instructions. For Northern analysis, RNA was isolated from exponential phase (OD_{600nm} ~ 0.4) cultures using RNAwiz™ (Ambion, Austin, TX) according to the manufacturer's protocol. Prior to electrophoresis, contaminating DNA was removed from the RNA prep using DNA-*free*™ DNase (Ambion). A total of 10 µg RNA per lane was separated in a gel of 1.2% (wt/vol) agarose with 1X formaldehyde- MOPS-EDTA sodium acetate buffer (Sigma, St. Louis, MO). Following electrophoresis the RNA was transferred to Zeta-Probe® membrane using a downward transfer method described in Ambion technical bulletin 169. The RNA was then permanently affixed to the membrane via UV crosslinking at 120 millijoules/cm^2.

Southern and Northern hybridizations were performed overnight at 42°C using 5 µl ^{32}P- labeled PCR products in ULTRAhyb® solution (Ambion). Blots were washed two times in 2X SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7)/0.1% (wt/vol) SDS for 5

min and two times in 0.1X SSC/0.1% (wt/vol) SDS for 15 min before placing on film. Probes for both Southern and Northern analyses were generated via PCR (see Table 3 for primer sequences and product sizes) and labeled with α-^{32}P-dCTP using the Prime-It® II Random Primer labeling kit (Stratagene).

Phenotypic analysis. Unless otherwise noted, 2% (vol/vol) subcultures from active cultures in standard Yen45 medium were used as the inoculum for phenotypic analyses. Response to iron limitation was monitored via optical density at 600 nm in Yen45 medium modified by omission of all known sources of iron and addition of 6, 3, or 1 μM added $FeCl_2$. The inocula for testing the growth responses to limiting iron were from medium containing 1 μM added $FeCl_2$. Modified Yen45 lacking added iron was analyzed by inductively coupled plasma spectrometry by the University of Missouri Agriculture Experiment Station Lab and found to contain 0.3 μM iron. To determine resistance to $MnCl_2$, colony forming units were determined following a 112-h exposure to 20, 30, or 40 mM $MnCl_2$. Growth responses to high concentrations of nitrate and nitrite were monitored via optical density of LS4D cultures amended to contain 50 or 100 mM nitrate or 2 or 5 mM nitrite at time 0. Responses to increased osmolarity were determined via optical density and microscopic observations following the addition of 300 mM NaCl or KCl (46) at the time of subculture. Osmoprotection was assayed by the addition of 2 mM glycine betaine at time 0 to subcultures containing 300 mM NaCl or KCl.

Microarrray analysis. Volumes of 600 ml of *D. vulgaris* Hildenborough wild type or JW707(Δ*fur*) were grown in LS4D as 100 ml batches in six 125-ml bottles to a cell density of ca. $3x10^8$ cells/ml (OD_{600} ~0.4). The 100 ml aliquots were used as inocula

into six replicate bottles each containing 900 ml LS4D and the cultures grown to log phase at 30ºC in anaerobic chambers. At log phase (OD_{600} = 0.38 for WT; 0.33 for JW707), 250 ml of each culture was harvested for sampling. For iron-limited experiments, the same protocol was followed with modified LS4D containing 5 μM $FeCl_2$ instead of 60 μM. Cultures were sampled at log phase, OD_{600} = 0.13 for WT and 0.17 for JW707. Cell harvesting, RNA extraction and microarray analyses were carried out as described previously (46).

RESULTS

Mutagenesis of *D. vulgaris fur* gene. Plasmid pMO707 (Fig. 1) was transferred to wild type *D. vulgaris* via electroporation. Positive (pSC27) and negative (lambda DNA) control DNAs were also electroporated using 10^9-10^{10} recipient cells for each transformation. To select for transformants, cells were plated on LS4D medium modified to contain 0.2% (wt/vol) yeast extract and 400 μg G418/ml and colonies appeared after four days at 30°C. The transformation efficiency for pMO707 was ca.1.2 x 10^{-7} per recipient cell, while the efficiency for the stable plasmid pSC27 was 1.4 x 10^{-6} per recipient cell. No G418 resistant colonies resulted from plating the negative control transformation. Colonies from the pMO707 transformation were subcultured into liquid medium of the same composition as that used for plating and a second single-colony isolation was made. Deletion of the *fur* gene via marker exchange was verified by a change in the size of a PCR product generated from primers complementary to regions up- and downstream of the *fur* gene (Fig. 2A). While the resulting PCR product from the

wild-type *fur* region was 2168 bp, the product from two selected transformants (JW706 and JW707) was 671 bp larger (2839 bp total). This difference correlates to the larger size of the Km^R determinant replacing the *fur* gene. Southern analysis with probes internal to *fur* or the Km^R determinant also corroborated replacement of the *fur* gene with the Km^R determinant (data not shown) and JW707 was selected for further analysis.

Northern analysis. RNAs from exponential phase (OD_{600nm} ~ 0.4) wild type and JW707 cultures grown in standard LS4D medium were analyzed for the expression of the *fur* gene as well as other genes predicted to be regulated by iron (61): *fld* (DVU2680)– flavodoxin, *sodB* (DVU2410) – superoxide dismutase, and *feoA* (DVU2572) – ferrous iron transporter (Fig. 2B). Hybridization analysis with the 334-bp *fur* probe indicated the presence of a single ~500 bp transcript for the wild type, but as expected, no signal was present in the JW707 lane (Fig. 2B, *fur* probe, lane 2). This transcript size correlates with the predicted *fur* ORF including a possible promoter sequence. For the *fld* gene, a weak band corresponding to a ~650 nt transcript was visualized for the wild type, while an intense band was present for JW707. While the *fld* ORF was annotated to be 447 bp, the Fur binding site was predicted to be 182 bp upstream (61). This hybridization signal is indicative of a sharp increase in expression. No transcript was evident for the *sodB* gene in either the wild type or JW707 samples, indicating expression at a level below detection (Fig. 2B). Results from the *feoA* probe indicated a large signal smear for JW707, with limited hybridization for the wild type. This lack of hybridization specificity may be due to the operon of which *feoA* is a part (DVU2571-2572), as well as the two genome copies of the *feoA* gene (DVU2572 and DVU2574). Predicted operon arrangements for the targeted genes are indicated in Table 4.

Response to iron. Growth curves for both wild type and JW707 in Yen 45 (30 μM $FeCl_2$) were identical, indicating that the *fur* gene is not essential for anaerobic dissimilatory sulfate reduction by *D. vulgaris* under iron replete conditions (Fig. 3A). To test the effect of iron limitation on the *fur* mutant, growth was analyzed in Yen 45 medium containing 1 and 6 μM added $FeCl_2$. Inocula used in this experiment were 2% (v/v) of active cultures in medium containing 1 μM added $FeCl_2$ to limit carry over of excess iron. Resulting growth curves for both iron levels were similar between wild type and JW707 (data not shown). The Fur mutant did not exhibit a growth advantage over the wild type when iron was limiting.

Metal sensitivity. Since a common trait of bacterial Δ*fur* mutants is increased resistance to Mn(II) (8, 30, 32, 40, 55), the effect of increasing concentrations of $MnCl_2$ on the growth of both wild type and JW707 strains was tested. Excessive precipitation caused by the addition of $MnCl_2$ to the growth medium (data not shown) mandated that CFUs be monitored as an indicator of Mn(II) effects on growth. Following 112 hours of exposure to 20, 30, or 40 mM $MnCl_2$, cultures were plated on Yen 45 medium. Wild-type untreated cultures had more CFUs than untreated JW707; however, with 40 mM $MnCl_2$, the number of wild-type CFUs declined from 1.3×10^8 to 1.4×10^5 (Fig. 4). By comparison, the CFUs for JW707 achieved in cultures exposed to $MnCl_2$ were similar, with 3.7×10^7 CFUs in the untreated control and 1.8×10^7 CFUs after exposure to 40 mM $MnCl_2$ (Fig. 4).

Response to osmolarity shock. Previous studies with wild-type *D. vulgaris* indicated that salt stress reduced growth rate and increased cell length and that these effects could be countered by addition of the osmoprotectant glycine betaine (46).

Interestingly, the putative Fur regulon members were among the most highly differentially transcribed genes during stress with NaCl or KCl (46) Therefore, the response of the *Δfur* mutant to salt stress was tested. Growth curves of cultures in medium supplemented with 300 mM additional NaCl or KCl (Fig. 3 B and C, respectively) were similar and growth rates decreased for both wild type and JW707. Microscopic observations of the salt-stressed cultures showed 91.4% ± 2.8% (S.D.) of wild type cells were elongated from 1.5 μm to 5 μM or more. Although JW707 cells were not counted, the fraction elongated appeared to be similar to the wild type. Addition of 2 mM glycine betaine with the salt protected the wild type from the decrease in growth rate (Fig. 3 B and C) and restored the cell morphology to normal. However, the growth rate of JW707 remained 75% of the uninhibited regardless of the presence of 2 mM glycine betaine. Interestingly, microscopic observations indicated that the osmolyte apparently restored the mutant cell lengths to ~1.5 μM (data not shown).

Response to nitrate/nitrite. Tests of JW707 growth responses to nitrate or nitrite stress were prompted by data from proteomic and microarray analyses of wild type *D. vulgaris* (35, 59). The transcript analyses indicated upregulation of both the *fur* regulon and genes predicted to be involved in iron-binding, particularly under nitrite stress. Significant differences in growth between wild type and JW707 were not observed in Yen 45 medium containing 50 mM $NaNO_3$ (data not shown). Increasing this $NaNO_3$ concentration to 100 mM did not have an effect on the final growth extent, but a 60 h lag phase for the wild type and an 80 h lag phase for JW707 did occur (data not shown). Growth of the wild type in Yen 45 medium supplemented with 2 mM $NaNO_2$ resulted in a lag phase of 60 h, whereas no growth was detected in medium supplemented with 5

mM $NaNO_2$ after 117 hours (Fig. 3D). In contrast, both 2 and 5 mM $NaNO_2$ completely inhibited the growth of JW707. Since comparable cell numbers were used as inocula for the parent and mutant, the results indicate an increased sensitivity to nitrite by the Δ*fur* mutant.

Expression profile of JW707. Transcriptional arrays covering 3,482 of the 3,531 (98.6%) protein coding sequences in the *D. vulgaris* genome were used to identify genes affected by deletion of the *fur* gene. In addition, the effect of iron concentration on the global transcription of the *fur* mutant was also determined by performing two separate experiments with growth medium containing 60 μM and 5 μM added $FeCl_2$. Samples were taken at similar optical densities for both JW707 and the parental wild-type strain with differential gene expression calculated as $\log_2$ ratios ($\log_2$R) using the following formula: $\log_2$ (transcripts of JW707) – $\log_2$ (transcripts of wild type). Following normalizations for signal intensities (13) and sector-based artifacts, the significance of the ratios was calculated as a Z-score. Generally ratios ≥ abs 1.6 (≥3-fold change in expression) were selected for further analysis.

Evidence that Fur is a global regulator in *D. vulgaris* derives from the observation that changes in gene expression with *fur* deleted were identified in 12 different functional categories based on the annotation of The Institute for Genomic Research. Transcript analysis revealed 34 and 50 genes differentially expressed at least three fold in response to the *fur* deletion (when compared to wild type) under iron-replete (60 μM) and iron-limited (5 μM) conditions, respectively (Fig. 5A). Comparison of the two data sets indicated that expression levels for 13 genes were affected under both iron conditions (Table 4A). Under iron-replete conditions 30 genes were up-regulated and 4 genes were

down-regulated with 44% of the total population predicted to encode hypothetical or conserved hypothetical proteins. Under iron-limiting conditions 32 genes were up-regulated and 18 genes were down-regulated with 30% of the total population predicted to encode hypothetical or conserved hypothetical proteins.

When JW707 responses to limiting iron were compared to its responses in iron-replete conditions, 22 genes were increased in expression as compared to 22 decreased. In contrast, when wild type was limited for iron, 50 genes were increased in transcription with only 18 decreased relative to iron-replete cells (Fig. 5B). Thus the genes differentially expressed in JW707 may be candidates for iron regulation. Those genes expressed differently in the wild type should include both those regulated by Fur and by iron concentration. Curiously only three genes meeting the stringent criterion of a three fold increase in expression were common to the iron-restricted JW707 and iron-restricted wild type: annotated as rubrerythrin (*rbr2*, DVU2318), chemotaxis protein (*cheY-2*, DVU2073) (Table 5, Category IV), and an ABC transporter permease protein (DVU2385) (Table 4).

Transcriptional profile of the predicted Fur regulon. Computational analysis of the upstream regions of *feoAB* operons from multiple δ-proteobacterial genomes revealed a conserved 17-bp palindromic motif corresponding to a putative Fur binding site Rodionov, 2004 #475}. A genomic scan of the *D. vulgaris* genome with this motif revealed 17 sites found upstream of eight genes or operons, constituting the putative Fur regulon (61). The prediction that these sites are operator sites for Fe(II)-bound Fur appears to have been supported by some of the transcriptional responses of the wild type when compared to those of the Fur mutant (asterisked genes in Table 4). Comparison of

the transcripts from the wild type under iron-limited versus iron-replete conditions also supported the prediction that Fur is not autogenously regulated in *D. vulgaris* (61)(data not shown). The lack of correlation between expression changes in the Fur regulon and the *fur* gene in microarray data from stressed *D. vulgaris* cells (35, 46) also suggests *fur* is not autogenously controlled.

The DVU2681 gene encoding a 60 amino acid hypothetical protein, exhibited the most up-regulation in the transcriptional profiling of JW707; $\log_2$ ratios of 5.31 and 2.30 under iron replete and restricted conditions, respectively. It should be noted that DVU2681 is located directly downstream of the flavodoxin gene (DVU2680) that is annotated as "iron-repressed" and, by Northern analysis, shown to be greatly increased in transcription in the Δ*fur* mutant (Fig. 2B). However, DVU2681 is transcribed in the opposite orientation. Other putative Fur regulated operons, such as those encoding the annotated GGDEF domain protein (DVU0763) and the HD domain protein (DVU3123) (Table 4B), did not appear to respond to decreased iron concentration. In the putative *genYZ* operon, only *genZ* (DVU0303) expression was consistent with iron-bound Fur repression. The latter two genes were predicted to be members of a Fur regulon unique to metal-reducing δ-proteobacteria though possible functions are unknown (61). In contrast, the hypothetical iron-regulated P-type ATPase gene (DVU3330) that was predicted to be monocistronic responds as if it were part of a larger operon of 2-4 genes (Table 4B). A similar expansion of Fur or iron influence on expression can be seen downstream of the *foxR* regulatory gene (DVU2378) that appears to include 12 genes (Table 4A). This cluster is composed of ABC transporters (DVU2380, DVU2384-87), the biopolymer transportors (TolRQ, DVU2388-89), and an energy generating mechanism for

transporting polymers across the membrane (TonB [DVU2390] and a TonB receptor [DVU2383]). While these genes have been shown to be involved in iron acquisition via siderophore production and transport in many bacteria (39, 44, 56), *D. vulgaris* has not been shown to produce siderophores (unpublished) nor have genes for siderophore biosynthesis been recognized in the genome. Whether the transcriptional changes are a direct response to Fur deletion or are mediated by influences on activities of other regulators, such as FoxR, will need to be addressed with further experimentation.

Other iron related genes. Transcriptional responses of genes that might be predicted to be involved with iron metabolism from the reported roles of their orthologs in other bacteria were examined (Table S1 in supplemental material). Expression profiles of a putative siderophore uptake system encoded by DVU0650-0646 and iron-storage proteins, bacterioferritin (DVU1397) and ferritin (DVU1568) did not support a role for Fur-regulation nor a clear response to iron concentrations. Two proteins requiring iron for function, Fe hydrogenase (DVU1771) and ferredoxin II (DVU0305), also were not significantly altered in expression in the absence of Fur.

Selected genes believed to be involved in the oxidative stress response of *D. vulgaris* were also examined for transcriptional responses in the deletion mutant (Table S1). Only the relative transcription for the gene annotated to encode alkyl hydroperoxide reductase C (DVU2247) was consistent with iron-bound Fur-dependent repression, although the changes did not meet our cutoff for significance. This gene product is reported to reduce hydrogen peroxide and to protect the cell from reactive oxygen species formed while iron acquisition systems are induced (inactive Fur repressor). Changes in the expression of genes for the putative cytochrome d ubiquinol

oxidase (DVU3270-71), superoxide dismutase (DVU2410), and catalase (DVUA0091) were not consistent with classical Fur-mediated regulation. As has been shown in other systems (4, 11, 25), the catalase gene transcription was increased by limiting iron, an example of regulation of a gene located on the megaplasmid in *D. vulgaris*.

Other potential modes of maintaining iron homeostasis. Iron responsive regulation through Fe(II)-bound Fur repressor is the most commonly identified microbial mechanism for maintaining iron homeostasis (31). However, a second Fe(II)-responsive repressor, DtxR, has been identified, primarily in Gram-positive bacteria (31), and a role for Fe(III)-specific two component regulatory systems has been recognized (56). From sequence analysis of *D. vulgaris*, homology to DtxR proteins has not been recognized nor have Fe(III)-specific histidine kinases and response regulators been annotated. However, the expression of a number of genes would be consistent with alternative modes of regulation (Table 5) as have been shown recently for *Helicobacter pylori* (15, 21). Category I illustrates the predicted pattern for Fe(II)-bound Fur regulation: increased expression in the absence of Fur in plentiful iron and when Fe(II) is limiting in wild-type cells. Other candidates are shown in Table 4. The two hypothetical genes shown in Category I (Table 5) are highly regulated but possible functions remain obscure. Category II is exemplified by amino acid biosynthesis genes for tryptophan and for methionine. These genes are up-regulated in the absence of Fur and low iron and decreased in expression in the presence of Fur and low iron, a pattern consistent with repression by iron-free Fur. Category III lists a few examples of genes that exhibit expression patterns that could be compatible with iron-free Fur induction. In limiting iron, low levels of expression are observed in the absence of Fur, but high levels of

expression are measured when Fur is present. In wild-type cells with 5μM $FeCl_2$, Fur would induce but the addition of 60 μM $FeCl_2$ would prevent induction. The apparent positive regulation by iron-free Fur in *D. vulgaris* requires confirmation before mechanisms are sought.

Regulation in response to iron concentrations that is not mediated by Fur may also be apparent in *D. vulgaris.* Categories IV and V show transcription patterns that seem consistent with iron repression and iron induction, respectively. For iron repression, candidate regulated genes were increased in expression in limiting iron regardless of the presence of Fur. For iron induction, the opposite pattern of transcript levels was seen. The two genes found increased in expression over three fold in both iron limited and iron replete conditions, *rbr2* and DVU2541, are among those shown in Category IV (Table 5). Curiously, Category V includes several genes encoded on the 200 kb megaplasmid present in the wild type *D. vulgaris*.

DISCUSSION

Genetic system. Here we describe a variation on the genetic system for *D. vulgaris* (26). We constructed the knockout cassette by fusion PCR to delete target genes via double recombination following electroporation. The development of this gene deletion method for *Desulfovibrio* was necessitated by a gene reversion event observed following plasmid interruption of the *cycA* gene in *D. desulfuricans* G20 (57). Due to the possibility of verifying deletion mutants within two weeks following cassette construction, this method can be used for rapid production of *D. vulgaris* deletion mutants. In 1997 Fu and Voordouw described the first case of targeted gene deletion in

D. vulgaris (26). This method is dependent on Cm^R selection/SacB counterselection following two separate recombination events and has been successful in generating at least 11 *D. vulgaris* deletion mutants (5 and references therein, 75). Because the SacB counter-selection procedure involves DNA transfer by conjugation as well as lengthy transformant screening, its use in high-throughput mutant construction is not practical.

A clean selection method is also key for the success of a genetic system and has been problematic in *Desulfovibrio* due to broad range antibiotic resistance. While the method described by Fu and Voordouw utilizes the Cm^R determinant (26), chloramphenicol resistance has been problematic in our hands. We have found that 400 μg of G418 (gentacin) per ml of LS4D medium provides clean selection when using the Km^R determinant. The utility of this method is illustrated in the 1.25×10^{-7} transformation efficiency obtained and the successful deletion of the *fur* gene in all three of the transformants screened via phenotypic ($MnCl_2$ resistance) and molecular (PCR and Southern analysis) methods.

The method described here is also dependent on DNA delivery by electroporation, which had not been successful in *D. vulgaris* until now. We added a high concentration of deletion vector as well as lambda DNA to an electroporation procedure first described for *Desulfovibrio fructosovorans* (64). The intrinsic nucleases of *D. vulgaris* likely linearize the plasmid and promote double recombination events and thus marker exchange between the vector and genome (5). Other deletion mutants screened in our laboratory using this deletion procedure follow the same pattern (data not shown).

Physiology of JW707. The similar decrease in growth rate for both the wild type and JW707 with 5 μM added $FeCl_2$ indicated that both cultures were limited for iron, but

not differentially (Fig. 3). Thus regardless of iron concentration, growth of the Δ*fur* mutant was neither inhibited nor promoted by the derepression of putative ferrous iron uptake genes (*feoAB*) (Fig. 2 and Table 4). Similarly, neither *Dichelobacter nodosus* or *Shewanella oneidensis* Fur mutants exhibited a detectable phenotype when grown anaerobically (52, 71). Without oxygen present, toxic radical formation resulting from increased ferrous iron and the Fenton reaction would be less likely. However, further tests are needed to determine if the cellular iron content of the *D. vulgaris* Fur mutant differs from wild type.

Osmotic stress. Salt stress in *Bacillus subtilis* has been shown to induce iron acquisition systems, therefore salinity has been proposed to cause iron-limitation (37, 70). Analysis of the *D. vulgaris* transcriptome and proteome under both NaCl and KCl stress indicated the same phenomenon, Fur regulon induction in response to salt stress (46). In contrast, physiological studies indicated growth inhibition of JW707 equivalent to the wild type when exposed to 300 mM NaCl or KCl (Fig. 3). Therefore, increased expression of the Fur regulon is not sufficient for overcoming this osmotic stress in *D. vulgaris*.

NO_3^-/NO_2^- stress. The effect of nitrate on *D. vulgaris* growth is of specific concern for environmental bioremediation applications. High levels of nitrate have been documented in uranium-contaminated sites (http://www.esd.ornl.gov/nabirfrc/) (20) and nitrate is believed to have an inhibitory affect on sulfate reduction through the intermediate nitrite (42, 49, 50). A previous proteomic analysis of wild type *D. vulgaris* showed that the presence of 105 mM $NaNO_3$ induced proteins involved in the ionic stress

response (59), although transcriptional profiling has indicated a response unique to nitrate that is not a composite of salt and nitrite (unpublished).

The increased sensitivity to nitrite stress for JW707 was unexpected based on previous transcript analyses of wild type *D. vulgaris* (35). An increased expression of the Fur regulon in the wild type was observed under nitrite stress (35). Therefore derepression of the regulon through deletion of the repressor was expected to provide an advantage for JW707 when exposed to nitrite. However, the Fur mutant was completely inhibited by 5 mM $NaNO_2$ (Fig. 3D). Like JW707, an *E. coli* Δ*fur* mutant was also more sensitive to $NaNO_2$ regardless of the upregulation of genes involved in iron uptake (48). This increased expression of the Fur regulon following 1 mM $NaNO_2$ treatment was hypothesized to be a consequence of NO nitrosylation of the Fe(II) in Fur that inactivated the repressor and likely other iron-containing genes (14). Direct evidence for this mechanism of iron modification in nitrite-treated *D. vulgaris* remains to be obtained.

Transcriptional profile of JW707. While the overall physiology of *D. vulgaris* was not dramatically affected by deleting the *fur* gene, a diverse transcriptional response occurred for JW707 when compared to the wild type. In many bacteria Fur negatively regulates siderophore production and transport (67 and references therein). Despite the up-regulation of a 12-gene cluster containing genes predicted to be involved in a siderophore uptake (*e.g.*, TolRQ; Table 4), no genes for siderophore production have been annotated in the *D. vulgaris* genome. This may allow *D. vulgaris* to save energy by simply stealing iron-complexed siderophores produced by other bacteria, as suggested for the spirochete *Leptospira biflexa* (43). In other bacteria, similar TonB/ABC and biopolymer uptake systems have also been shown to transport vitamin B_{12}, phage,

colicins, and maltodextrins into the cell (51, 54). Further studies are needed to determine what role the large 12 gene cluster plays in *D. vulgaris.*

The high level of differential expression for the *feoAB* operon, apparently regulated by Fur and its corepressor Fe(II) (Fig. 2 and Table 4), is interpreted as evidence that the FeoAB system is the primary iron uptake mechanism in *D. vulgaris*. This follows from the prediction that Fe(II) predominates in anaerobic environments. However, genes predicted to be involved in iron storage such as *bfr* and *ftn* did not exhibit a strong transcriptional response to either iron concentration or the deletion of the *fur* gene.

Oxidative stress. The Fur regulator has been shown to be intricately involved in the oxidative stress response of some bacteria (2, 33, 34). Thus, it can be argued that regulation by Fur is linked to an increased uptake of iron that can generate reactive oxygen species upon oxygen exposure. Despite the anaerobic lifestyle of *D. vulgaris*, it is known to survive exposure to oxygen and contains several oxidative stress response genes within its genome. As such it was not surprising to find *ahpC*, *rdl*, *cydA*, and *katA* genes were differentially expressed at low levels in this study. However, only *ahpC* appeared to be classically regulated by iron-bound Fur (data not shown). The diverse regulatory pattern of the other oxidative stress response genes may be explained by co-regulation by PerR, a homolog to Fur that also responds to iron concentration (9, 11). In fact *ahpC* and *rdl* are predicted to possess a PerR binding site within their promoter region (61). Co-regulation of *katA* and *ahpC* by Fur and PerR has also been proposed in the microaerophilic *Campylobacter jejuni* (3, 38, 73). Further studies are needed to determine if Fur and PerR regulation overlap in *D. vulgaris*.

Other mechanisms of Fur regulation. In addition to the traditional repressor role of iron-bound Fur, alternative activation and repression by iron-free Fur were inferred from the transcription patterns seen in *D. vulgaris*. These two forms of regulation have recently been described in *Helicobacter pylori* (15, 18, 22). While iron-bound Fur has been shown in the literature to be a positive regulator, it is possible that some of the affected genes are actually repressed by iron-free Fur. This form of Fur regulation was described for the *pfr* (encodes a ferritin) and *sodB* genes of *H. pylori* (18, 22). Genes for which iron-free Fur repression is consistent in *D. vulgaris* are the 7 genes of the tryptophan operon and *metK* (Table 5 II).

Alternatively, the transcription of flagellar genes (DVU1443-1445) appeared to be consistent with induction by iron-free Fur in *D. vulgaris* and DVU1444 expression changes met the criteria used for an example in this category (Table 5 III). This form of positive regulation by iron-free Fur has only been reported for *flaB,* a major flagellin gene, in *H. pylori* (15). It is tempting to infer that motility may be decreased in the presence of plentiful iron for both *D. vulgaris* and *H. pylori*. Another example of positive regulation by Fur is activation by iron-bound Fur, suggested for a collection of *Neisseria meningitidis* genes involved in both aerobic and anaerobic respiration (17). However, expression patterns suggesting this form of regulation were not observed in this study.

Iron regulation independent of Fur. Differential expression of genes that is independent of Fur implies that other regulators may respond to iron concentrations within the cell. Possible examples of these regulators include PerR, DtxR, NikR, Irr, and RirA (6, 9, 10, 15, 16, 28). However, only *perR* has been annotated in the *D. vulgaris* genome (61) with DVU2318 (encoding a rubrerythrin) being the only gene in the iron-

responsive category predicted to be PerR regulated (Table 5 IV) (61). Thus it appears that other iron-responsive regulators may be present in *D. vulgaris*, but have not yet been identified. Based on the iron-dependent regulation of megaplasmid encoded genes (Table 5 V), these iron-responsive regulators may not be limited to the *D. vulgaris* chromosome, but may also be present on the megaplasmid. However, preliminary searches for regulatory motifs upstream of genes showing similar differential expression patterns have been unsuccessful. This is not too surprising because of the small number of candidate genes involved in each category, although both the genes from *D. vulgaris* and the orthologs from *D. desulfuricans* G20 were included in the search (data not shown).

In summary, both the physiological and microarray data indicate a global regulatory role for Fur in *D. vulgaris,* including involvement in the osmotic and nitrite stress responses. While a traditional repressor role for iron-bound Fur was observed, expression data also suggests activation by iron-free Fur. The patterns of apparent transcription regulation presented here require further confirmation but indicate that alternate models of iron homeostasis may be functioning in *D. vulgaris* and possibly anaerobes in general. Specifically, studies to determine the roles of iron-free Fur and the TonB/TolQR system in addition to identification of other iron-dependent regulators are needed. These issues are especially pertinent because of the differential expression of the *D. vulgaris* Fur regulon upon exposure of the cells to various environmental stressors (35, 46, 74). Information from these studies may prove integral in the design of future bioremediation strategies for the removal of heavy metals.

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FIGURE LEGENDS

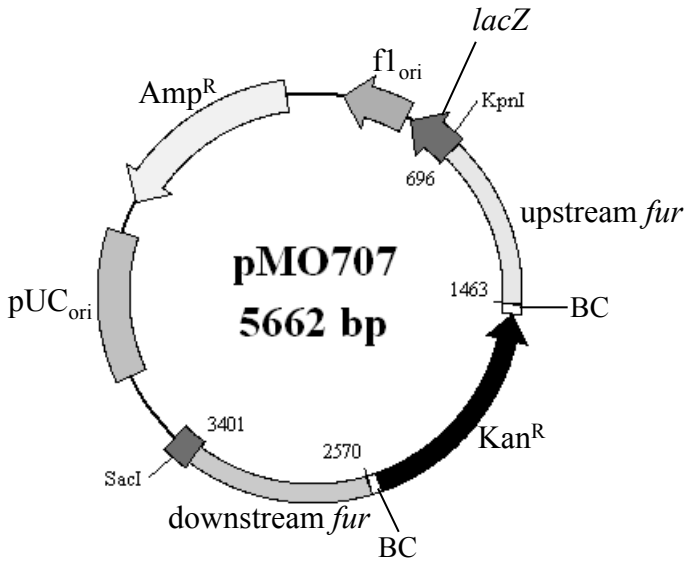
Figure 1. Map of pMO707 containing the *fur* deletion construct. A 2706 bp PCR product containing the pSC27 KanR determinant flanked by DNA sequences upstream (768 bp) and downstream (831 bp) of the *D. vulgaris fur* gene was inserted into the *Eco*RV site of the pBluescript (SK+) multi-cloning site. Both pBluescript and pMO707 are unstable in *D. vulgaris*. Numbers indicate position of sequences upstream and downstream of the *fur* gene; BC indicates molecular barcodes allowing mutant tracking in a mixed population.

Figure 2. A) PCR analysis of Km^R *D. vulgaris* transformants using primers targeting regions outside of the *fur* knockout cassette (P7/P8). Lane 1, wild type; lane 2, transformant A; lane 3, transformant B; lane 4, pMO707; lane 5, no DNA; lane M, 1 kb marker. An increase in product size from 2168 bp to 2839 bp indicates exchange of the *fur* gene for the Km^R determinant creating JW707. B) Northern analyses of total RNA (10 μg) from JW707: Lane 1, wild type; lane 2, JW707; lane M, RNA marker. Probes used for hybridization are indicated above blot.

Figure 3. Growth curves of *D. vulgaris* wild type (open symbols) and mutant strain JW707 (filled symbols) under various conditions. **A)** Iron replete (30 μM $FeCl_2$); **B)** Response to osmolarity stress, sodium as 300 mM NaCl (circles) or 300 mM NaCl plus 2 mM glycine betaine (squares). **C)** Response to osmolarity stress, potassium as 300 mM KCl (circles) or 300 mM KCl plus 2 mM glycine betaine (squares). **D)** Response to nitrite at 2 mM $NaNO_2$ (circles), 5 mM $NaNO_2$ (squares). Curves are representative of three trials at 37°C.

Figure 4. Growth response of *D. vulgaris* wild type (open symbols) and mutant strain JW707 (filled symbols) to $MnCl_2$ treatment. Colony forming units following exposure to 0, 20 mM, 30 mM, and 40 mM $MnCl_2$. Curves are representative of three trials at 37°C.

Figure 5. A) Genes differentially expressed three fold in the Δ*fur* mutant, JW707, when compared to wild-type cells, in iron-replete ("+Fe," 60 μM) and iron-limited ("-Fe," 5 μM) conditions. B) Overlap of genes differentially expressed three fold in JW707 iron-limited ("-Fe," 5 μM) conditions compared with JW707 iron-replete conditions versus those differentially expressed in iron-limited wild-type cells compared to iron-replete wild type cells.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KpnI
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Kan^R
SacI
downstream fur
BC

Figure 1

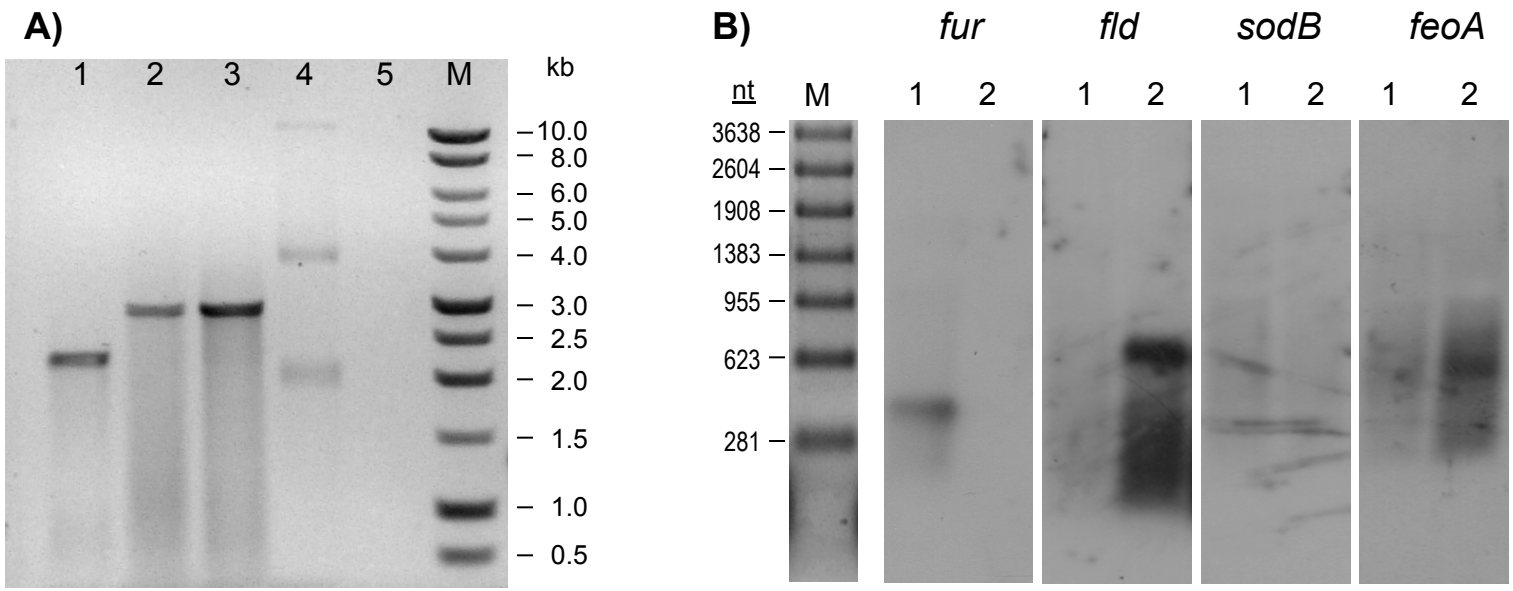
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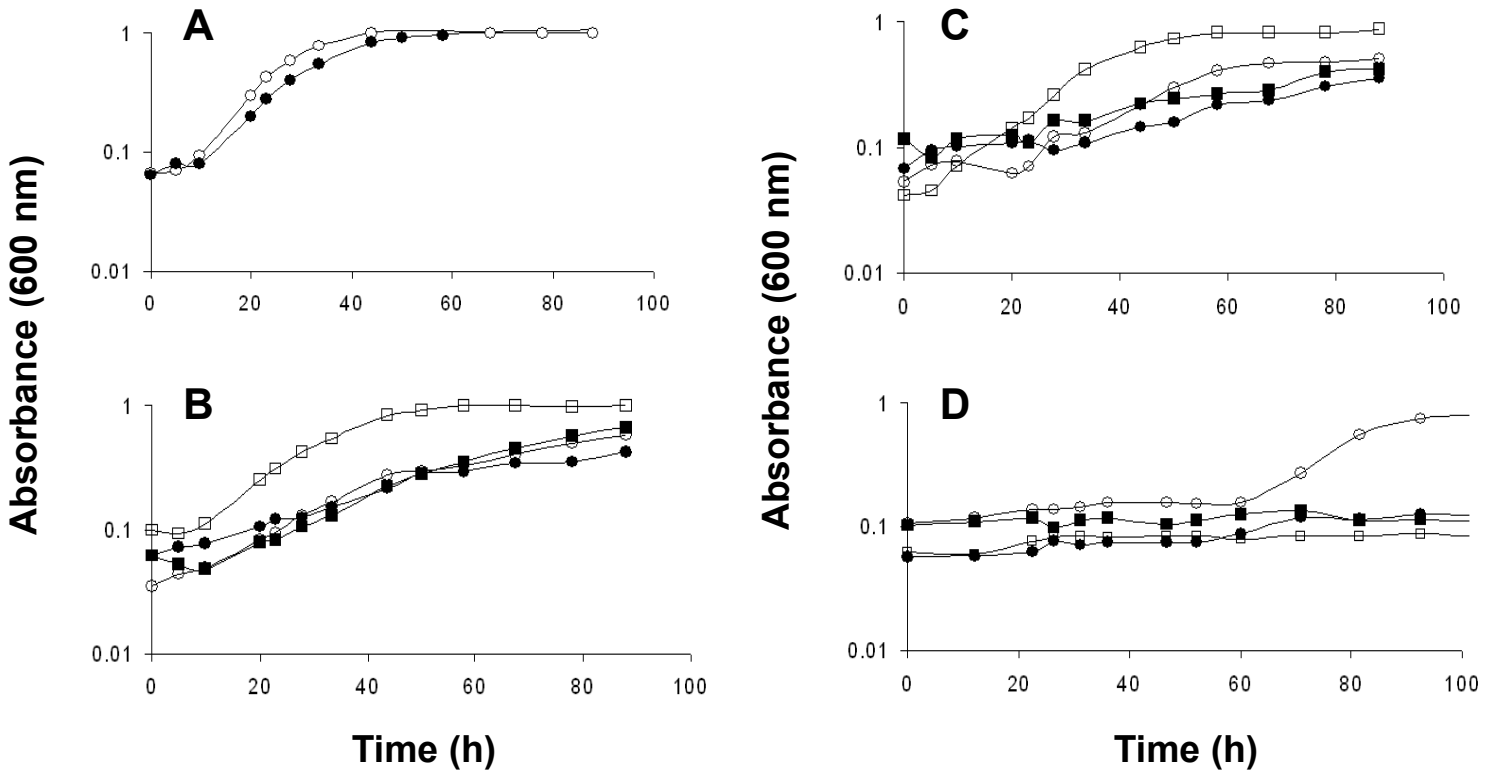
Figure 2


A
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Absorbance (600 nm)
Absorbance (600 nm)
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Figure 3

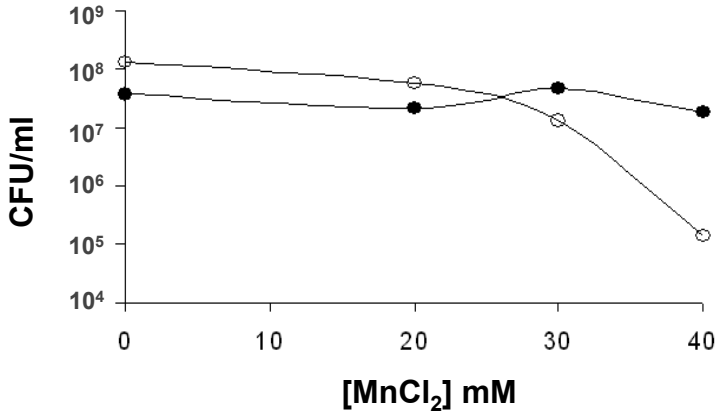

Figure 4

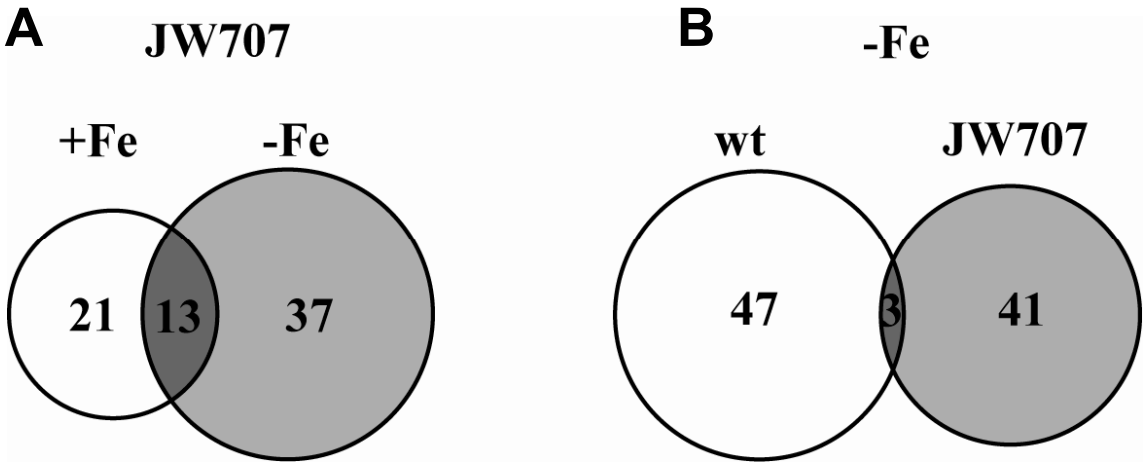

Figure 5

TABLE 1. Bacteria and plasmids used in this study.

Bacterial Strain	Description of relevant features	Reference
Desulfovibrio vulgaris strain Hildenborough	wild type, NCIMB 8303	(36, 53)
D. vulgaris JW707	*Δfur*, Km^R	this study
Escherichia coli JM109	Cloning strain	Promega
Plasmid		
pBluescript (SK+)	blue-white cloning vector, Amp^R	Stratagene
pSC27	*Desulfovibrio* shuttle vector; source of Km^R cassette	(63)
pMO707	pBluescript containing 2,839 kb *fur* deletion cassette: Amp^R, Km^R	this study

TABLE 2. PCR primers for deletion cassette construction.

Primer	Sequence 5'- 3'	Position of the 5' end
P1	CTCTCTGCAACCTGACGGCG	-780 bp to *fur* ORF
P2	AAGACTGTAGCCGTACCTCGAATCTACTCTTGGGTTGGTGTCTGTGC	-13 bp to *fur* ORF
P3	AATCCGCTCACTAAGTTCATAGACCGAAGCGCATGTGAAGACTGCTGT	+5 bp to *fur* ORF
P4	GCACGAAGGACTCGAGAGGATTCGATGTC	+835 bp to *fur* ORF
P5	TAGATTCGAGGTACGGCTACAGTCTT**GTGGACTGACGGCTAATCTC**CCCCAGAGTCCCGCTCAG	+14 bp to Km^R ORF
P6	CGGTCTATGAACTTAGTGAGCGGATT**CGTAACGGCTTGTACCCAGA**GAGGTAGCTTGCAGTGGGCT	-205 bp to Km^R ORF
P7	GTGCGCGACGACCTTGAAA	-832 bp to *fur* ORF
P8	TGCGGCCTCGAACTTCGACT	+901 bp to *fur* ORF

Underlining indicates common sequences used for PCR.
Bold indicates unique barcoding sequences.

TABLE 3. PCR primers for gene probe construction.

Target	Primer	Sequence 5'- 3'	Product (bp)
DVU0942: *fur*	fur-65F	AGCGGATGCTCATCGTCGAT	334
	fur-399R	GAGATACATCCGGTGCGAGG	
DVU2680: *fld*	fld-16F	ATCGTCTACGGTTCCACCAC	294
	fld-310R	CCCCGCAGAAGTACTCGTAG	
DVU2410: *sodB*	sodB-88F	GCCTACTACGCCAACCTCAA	400
	sodB-488R	CATACGTCGATGGTCAGCAC	
DVU2572: *feoA*	feoA1-13F	ATCTCCCTGCGGCAGTTG	230
	feoA1-243R	GTCGGCTTCCATCACTTCAA	
Km^R from pSC27	Km-intF	AGATCTGATCAAGAGACAGGATGAG	729
	Km-intR	CTCTTCAGCAATATCACGGGTAG	