Identification of a *Dichelobacter nodosus* Ferric Uptake Regulator and Determination of Its Regulatory Targets

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The expression of iron regulated genes in bacteria is typically controlled by the ferric uptake regulator (Fur) protein, a global transcriptional repressor that regulates functions as diverse as iron acquisition, oxidative stress, and virulence. We have identified a *fur* homologue in *Dichelobacter nodosus*, the causative agent of ovine footrot, and shown that it complements an *Escherichia coli fur* mutant. Homology modeling of the *D. nodosus* Fur protein with the recently solved crystal structure of Fur from *Pseudomonas aeruginosa* indicated extensive structural conservation. As Southern hybridization analysis of different clinical isolates of *D. nodosus* indicated that the *fur* gene was present in all of these strains, the *fur* gene was insertionally inactivated to determine its functional role. Analysis of these mutants by various techniques did not indicate any significant differences in the expression of known virulence genes or in iron-dependent growth. However, we determined several Fur regulatory targets by two-dimensional gel electrophoresis coupled with mass spectrometry. Analysis of proteins from cytoplasmic, membrane, and extracellular fractions revealed numerous differentially expressed proteins. The transcriptional basis of these differences was analyzed by using quantitative reverse transcriptase PCR. Proteins with increased expression in the *fur* mutant were homologues of the periplasmic iron binding protein YfeA and a cobalt chelatase, CbiK. Down-regulated proteins included a putative manganese superoxide dismutase and ornithine decarboxylase. Based on these data, it is suggested that in *D. nodosus* the Fur protein functions as a regulator of iron and oxidative metabolism.

Dichelobacter nodosus is a fastidious gram-negative anaerobe that is the causative agent of footrot in sheep, goats, and other ruminants. Ovine footrot is a debilitating disease that results in the hoof separating from the underlying soft tissue, leading to lameness, loss of condition, and reduced wool growth. The three recognized forms of the disease, virulent, intermediate, and benign, differ in their clinical pathologies, with less virulent isolates causing a lower percentage of severe lesions under optimal climatic conditions (54). The virulence of an individual strain depends on factors such as its type IV fimbriae, extracellular serine proteases, and potentially on two genomic regions, the virulence-related locus (*vrl*) and the virulence-associated protein (*vap*) regions, that are preferentially associated with virulence (7, 34). Until recently, the molecular analysis of *D. nodosus* has been hindered by a lack of genetic tools, but we have now developed methods for the genetic manipulation of *D. nodosus* (33, 34).

Iron is an important micronutrient and is essential for the growth of most living cells. The levels of iron available to a cell can vary greatly depending upon the immediate environment, and since excess levels of iron can be toxic due to the formation of oxygen radicals, the uptake of iron needs to be appropriately regulated (2). The expression of iron-regulated genes in bacteria is typically controlled by the ferric uptake regulator (Fur) protein. Fur is a small, 17-kDa, global transcriptional repressor that in the presence of iron regulates functions as diverse as iron acquisition, oxidative stress, and virulence (23). The co-

ordinated expression of virulence factors by iron availability is not surprising, since the concentration of iron at most host infection sites is low, due to stringently regulated transport systems. These low iron levels signal to bacteria that they have entered the host and subsequently lead to increased expression of iron acquisition systems as well as virulence factors. Furthermore, in some bacterial species, *fur* mutants have shown a reduction in virulence (29, 41, 47). Fur also acts to positively regulate genes involved in the acid tolerance response in *Salmonella enterica* serovar Typhimurium and regulates superoxide dismutase in *Escherichia coli*. The regulation of superoxide dismutase has been attributed to a small RNA molecule that is negatively regulated by Fur (2).

Fur is a zinc metalloprotein, rich in histidines, that contains one zinc and one iron binding site per monomer. It contains an N-terminal DNA recognition domain and a C-terminal domain that is involved in dimerization (16). The recent elucidation of the crystal structure of the Fur protein from *Pseudomonas aeruginosa* (Fur$_{Pa}$) pinpointed the location of the metal binding sites and provided an insight into the architecture of the Fur-DNA interaction (42). The N-terminal region contains a winged-helix motif that binds to regions of DNA referred to as Fur boxes that are normally located between the −10 and −35 promoter region of Fur-repressed genes (15, 42). DNA binding occurs when Fur is complexed with Fe^{2+}, and it is thought to inhibit the binding of RNA polymerase (16). The original *E. coli* Fur (Fur$_{Ec}$) box consensus consisted of a 19-bp palindromic sequence, GATAATGATAATCATTATC, which has now been refined to three hexameric repeats of 5′-NAT(A/T) AT-3′ (16).

Fur homologues have been identified in both gram-negative

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference
Strains		
E. coli		
DH5α	F⁻ *endA1 hsdR17* (r_k⁻m_k⁻) *thi-1* λ⁻ *recA1 gyrA96 RelA1 PhoA suipE44 deoR* φ80d*lacZ*ΔM15 Δ(*lacZYA argF*)*U169*	Invitrogen
H1698	*AraD139Δ* (*argF-lac*)*U169 rpsL150 relA1 FlbB5301 deoC1 ptsF25 rbsR fiu*::λpLacMu53	24
H1780	*AraD139Δ* (*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR fur fiu*::λpLacMu53	24
D. nodosus		
VCS1703A	Serogroup G, transformable, virulent strain	J. Egerton, University of Sydney
JIR3764	VCS1703A *fur*Ω*erm*(B)	Natural transformation
JIR3765	VCS1703A *fur*Ω*erm*(B)	Natural transformation
Plasmids		
pBluescript SK⁺	Apʳ, *lacZ* cloning vector	Stratagene
pUC18	Apʳ, *lacZ*, cloning vector	63
pJIR2236	pUC18 EcoR1/XbaIΩ(EcoRI/XbaI VCS1703A PCR product, *fur* gene ~700bp)	Recombinant
pJIR2281	pBluescript SK⁺ containing *E. coli fur* gene	Recombinant
pJIR2300	pBluescript SK⁺ with 2kb ClaI fragment containing *D. nodosus fur* gene	Recombinant
pJIR2417	pBluescript SK⁺ containing *erm*(B) flanked by N-terminal and C-terminal region of *D. nodosus fur*	Recombinant

and gram-positive bacteria. Many of these homologues are able to complement a *fur_{Ec}* mutant, demonstrating that the molecular mechanism underlying transcriptional regulation by Fur is highly conserved (16). However, little has been done to describe the role of Fur in an anaerobic organism. In this study we aimed to identify and determine the regulatory role of a Fur homologue from *D. nodosus*. We report the successful cloning and functional analysis of the *D. nodosus fur* gene, the construction of a *D. nodosus fur* mutant, and the use of proteomics to identify proteins whose expression in *D. nodosus* is Fur regulated.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains and plasmids are listed in Table 1. *E. coli* strains were grown at 37°C on 2× YT medium (yeast extract, tryptone, and sodium chloride) (51) supplemented with 100 μg of ampicillin per ml or 150 μg of erythromycin per ml. *D. nodosus* strains were grown at 37°C in an anaerobic chamber (Coy Laboratory Products, Inc.) in an atmosphere of 10% (vol/vol) H₂ and 10% (vol/vol) CO₂ in N₂ on Eugon (Difco) yeast extract (EYE) agar with 5% defibrinated horse blood (Equicell), supplemented with 1 μg of erythromycin per ml for the selection of transformants, or in Eugon (Difco) broth with yeast extract. Iron-replete and iron-limiting conditions were achieved by the addition of 100 μM ferric chloride and 50 μM deferoxamine mesylate (Desferal; Sigma) or 200 μM 2,2′-dipyridyl (Sigma), respectively.

Library screening. The *D. nodosus* library was screened by using H1780, a *fur_{Ec}* mutant containing an in-frame insertion of *lacZ* into the Fur-regulated *fiu* gene (24). In this strain expression of β-galactosidase is constitutive, and therefore red colonies are observed on iron-replete MacConkey (MAC) agar. If a *fur* gene is provided in *trans*, the translated protein should actively bind to the promoter and repress the transcription of *lacZ*, resulting in yellow-orange colonies.

Phenotypic analysis. Protease phenotypes were determined on EYE agar containing 2% (wt/vol) skim milk powder (34). The elastase test (55) and twitching motility assays (34) were performed as previously described. β-galactosidase activity was determined by using cultures treated with sodium dodecyl sulfate (SDS) and chloroform as previously described (40). Each assay was repeated at least four times with separate cultures. Statistical analysis was performed by using a Student's *t* test.

Molecular techniques. Unless otherwise stated, molecular techniques were performed by using standard procedures (51). Reverse transcriptase PCR (RT-

PCR) of *fur* was carried out as described (34) by using primers 16329 and 16441 (Table 2). Oligonucleotide primers (Table 2) were synthesized by using a 393 DNA-RNA synthesizer (Applied Biosystems). Sequencing was performed with an Applied Biosystems 373A automated sequencer and analyzed by using Sequencher version 3.0 (Gene Codes Corporation). Nucleotide and amino acid comparisons were accomplished by using the National Center for Biotechnology Information BLAST server (http://www.ncbi.nlm.nih.gov/BLAST) (1) and resources available at TIGR (http://www.tigr.org). Theoretical values for molecular mass and isoelectric points were determined by using the ProtParam tool located on the ExPASy molecular biology server (http://us.expasy.org). Signal peptides were identified by using SignalP (http://www.cbs.dtu.dk/services/SignalP).

Quantitative RT-PCR (QRT-PCR). RNA was isolated from *D. nodosus* cells grown in EYE broth with 100 μM ferric chloride, by using Trizol (Invitrogen) according to the manufacturer's instructions. RT reactions were performed as described previously (34) by using primers designed with Primer Express (Applied Biosystems) (Table 2). DNA amplification was determined by using the fluorescent dye SYBR Green. Reactions were performed in final volumes of 25 μl with the SYBR Green PCR master mix (Applied Biosystems), cDNA, and 50 nM primers on an ABI PRISM 7700 sequence detector. Samples were run in triplicate on multiple runs and calibrated against genomic DNA standards. The data were normalized to 16S rRNA levels, and reactions were the result of single products as determined by the analysis of dissociation curves.

Mutant construction and analysis. The *fur* suicide vector, pJIR2417, was constructed in a pBluescript SK⁺ derivative that contained the *erm*(B) gene. Fragments generated by PCR representing the 5′ *fur* region and its upstream sequence (primers 18162 and 18163) and the 3′ region of *fur* and its downstream region (18156 and 18164) were each cloned on opposite sides of *erm*(B) in the genomic orientation. Natural transformation of *D. nodous* cells was then performed as previously described (34), and a capillary PCR (33) was used with primers 16944 and 16875 to screen transformants for an appropriately sized insertion into the *D. nodosus fur* gene. Two mutants were then screened further by PCR with 16S rRNA gene primers (35) to confirm that the transformants were derived from *D. nodosus* and *erm*(B) primers (17) to verify that the *erm*(B) gene was present. Southern hybridizations were carried out as described (34) by using NruI (New England Biolabs)-digested chromosomal DNA isolated from wild-type and mutant strains with a *fur*-specific probe, constructed by using primers 16329 and 16441 (Table 2), an *erm*(B)-specific probe (17), and a 16S rRNA gene probe (35). HindIII (Roche)-digested DNA was hybridized with a *vap*-specific probe (31). To confirm that the antibiotic-resistant colonies were derived from the wild-type strain, Southern analysis with the 16S rRNA and *vap* probes, combined with PCR-restriction fragment length polymorphism analysis of the gene encoding an outer membrane protein of *D. nodosus* (*omp1*) (20) was performed.

TABLE 2. Oligonucleotides used in this study

Primer	Gene	Sequence (5′-3′)[a]	Use
16329	*fur*	CGTAATGGTGTTTTTGTGCAAT	RT-PCR, PCR
16441	*fur*	AAGAAATTGGTTTGGCAACG	RT-PCR, PCR
16661	*fur*	<u>GCTCTAGA</u>AAGTCTGGCAGGAAATACGC	PCR
16662	*fur*	<u>CGGAATTC</u>GCCTTGCCGTTGTAAATGTAA	PCR
16875	*fur*	CATGCCCGCCATCATTGTGC	PCR
16944	*fur*	GACAGATGAAAGCGTTGACC	PCR
18156	*fur*	GCAATTTGAACAAGCGGGCAT	PCR
18162	*fur*	<u>GGAATTC</u>CCAATTTCTTCATCGGCAGCC	PCR
18163	*fur*	<u>CCATCGAT</u>GAAAATTGCGACCACAAAGCG	PCR
18164	*fur*	<u>TCCCCCGCGG</u>CGCGCAGTTCAACACGAAAT	PCR
25946	16S rRNA	CGGAATGACTGGGCGTAAA	QRT-PCR
25948	16S rRNA	CTAGGTTGAGCCCAGGGATTT	QRT-PCR
25619	*sodA*	GCAGCGCTATCCGCAATAAT	QRT-PCR
25620	*sodA*	CGCATCGCGTTGAAATAAAGA	QRT-PCR
25606	*yfeA*	ACAGAAGGCGCTTTTTCCTATTTA	QRT-PCR
25621	*yfeA*	CGGCCACACATAGGCTTCA	QRT-PCR
25608	*cbiK*	AATGGGAAATGAATAAAACTAGAAAGGA	QRT-PCR
25604	*cbiK*	GCATGGTTGAAATACCGCAAT	QRT-PCR
25616	*speF*	CGCTCCGGGTGATATGGTT	QRT-PCR
25617	*speF*	GCACCGTGGCAAACAGATTT	QRT-PCR

[a] Underlining represents sequence for cloning purposes.

Homology modeling. The structure of the *D. nodosus fur* (Fur$_{Dn}$) protein was modeled on the crystal structure of the Fur$_{Pa}$ protein (42) by using SWISS-MODEL and DeepView (Swiss-PdbViewer) (22). The final model was verified by using a Ramachandran plot and various other analytical tools contained in the WhatIf suite of programs (http://www.cmbi.kun.nl/gv/servers/WIWWWI).

Preparation of proteins for proteomic analysis. Cell lysates were prepared from 150-ml cultures of *D. nodosus* grown for 24 h in iron-replete EYE broth. Cells were pelleted at 6,000 × g before being washed with 1× TE buffer (10 mM Tris–HCl[pH 8.0], 1 mM EDTA), resuspended in immobilized pH gradient (IPG) rehydration solution (8 M urea, 0.03% dithiothreitol [wt/vol], 2% [wt/vol] CHAPS [3-([3-cholamidopropyl]-dimethylammonio)-1-propanesulfonate], 0.5% IPG buffer [vol/vol; Amersham Biosciences], 0.002% [wt/vol] bromophenol blue) plus 1 mg of DNaseI (Promega) per ml and 0.25 mg of RNase (Roche) per ml, and subjected to three 30-s bursts of sonication (Sonifier B-12; Branson). Cellular debris was removed by centrifugation.

Membrane-enriched samples were obtained from 24-h, 150-ml *D. nodosus* iron-replete EYE broth cultures as previously described (10) before solubilization in IPG buffer, pH 4 to 7. To obtain secreted proteins, *D. nodosus* was grown in 150 ml of iron-replete EYE broth plus 1 mM CaCl$_2$ for 48 h. After centrifugation, 20 ml of the supernatant was filtered through a 0.45-μm-pore-size filter (Millipore) and then precipitated overnight at 4°C with 10% (wt/vol) trichloroacetic acid. Secreted proteins were pelleted at 10,000 × g before being washed with cold acetone and resuspended in IPG buffer, pH 3 to 10. Protein concentrations were determined by using a modified Bradford method (44).

Two-dimensional gel electrophoresis. Samples were loaded by passive rehydration onto 24-cm IPG strips (Amersham Biosciences) according to the manufacturer's instructions. Isoelectric focusing was carried out by using a gradient-based protocol for a total of 40 and 60 kVh for pH 3 to 10 and pH 4 to 7 IPG strips, respectively, by using a Multiphor II electrophoresis unit (Amersham Biosciences). Before SDS-polyacrylamide gel electrophoresis, IPG strips were equilibrated for 15 min in SDS equilibration buffer (6 M urea, 50 mM Tris–HCl [pH 8.8], 30% glycerol [vol/vol], 2% SDS, 0.002% bromophenol blue [wt/vol]) containing 65 mM dithiothreitol and then for 15 min in equilibration buffer containing 135 mM iodoacetamide. The strips were analyzed on large ExcelGel XL 12 to 14% gradient gels (Amersham Biosciences) for the cell lysate and membrane preparations or on smaller ExcelGel 12.5% homogeneous gels (Amersham Biosciences) for the extracellular proteins by using a Multiphor II apparatus according to the manufacturer's instructions (Amersham Biosciences). Two-dimensional gels loaded with analytical quantities of protein were visualized by silver staining as previously described (3). Proteins to be identified by mass spectrometry (MS) were stained with colloidal Coomassie brilliant blue G-250 according to the manufacturer's instructions (Sigma). Gel analysis was performed in triplicate, and only proteins differentially expressed across all replicates were identified by MS. Since proteomics had not previously been done in *D. nodosus*, proteins were initially resolved over a pH 3 to 10 gradient. This

process revealed that the majority of observable proteins were located in the acidic to neutral portion of the gel; therefore, further work was carried out by using a pI range of 4 to 7.

Identification of proteins by MS. The proteins were excised from a gel, and the gel slices were washed for at least 1 h in both double-distilled H$_2$O and then 50% acetonitrile, before dehydration in acetonitrile for 15 min. The gel pieces were lyophilized before rehydration overnight at 37°C with 25 mM ammonium bicarbonate containing 10 mg of sequencing grade trypsin (Promega) per ml. The supernatant from this step was retained while two further 1-h peptide extractions were performed with 50% acetonitrile–0.1% (vol/vol) trifluoroacetic acid (TFA). The peptide extracts were then pooled, lyophilized, and reconstituted in 1% TFA. The peptides were then concentrated and desalted by using C18 Zip Tips (Millipore), according to the manufacturer's recommendations, and eluted with 50% acetonitrile–0.1% TFA. Matrix assisted laser desorption ionization-time of flight MS was performed on a Voyager De STR mass spectrometer (Applied Biosystems) by using the crushed crystal method with α-cyano-4-hydroxycinnamic acid. Peptide mass fingerprint spectra were automatically submitted to MS-Fit, part of the ProteinProspector (9) package, and searched against a *D. nodosus* set of open reading frames (ORFs) that we predicted by using Glimmer (12) from our completed but unpublished genome sequence (http://www.tigr.org). Typically, a mass tolerance of 50 ppm was used in these analyses. Glimmer, based on interpolated Markov models, was trained with ORFs larger than 600 bp from the genomic sequence, as well as with the *D. nodosus* genes available in the GenBank database.

Nucleotide sequence accession number. The sequence of the *D. nodosus fur* gene has been deposited in the GenBank database under the accession number AY569334.

RESULTS

Cloning and sequence analysis of a *D. nodosus fur* homologue. As part of a larger study aimed at identifying response regulator genes by using degenerate oligonucleotide PCR on DNA from the transformable *D. nodosus* strain VCS1703A, we identified a recombinant plasmid, pJIR2236, that appeared to encode a product with similarity to the C-terminal 90 amino acids of Fur proteins. Southern blotting showed that this gene region was localized to a 2-kb chromosomal ClaI fragment. A chromosomal VCS1703A ClaI library was constructed in pBluescript SK$^+$ and then screened with the *E. coli* reporter strain H1780. Approximately 20 of the 40,000 colonies that were screened were pink on iron-replete MAC agar and red on
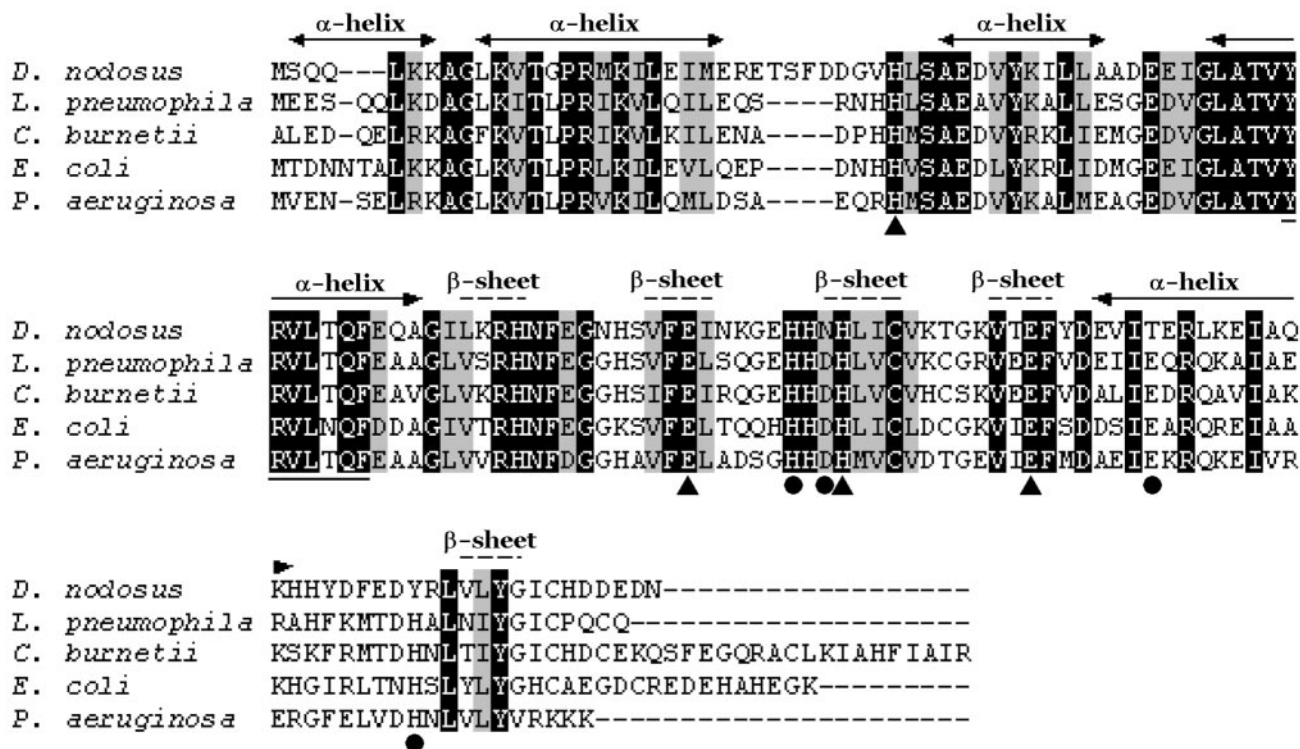
FIG. 1. Alignment of *D. nodosus* Fur homologues. The Fur$_{Dn}$ protein was aligned by using ClustalW (59) to its two highest scoring matches as determined by BLASTP (1) as well as to the *E. coli* and *P. aeruginosa* Fur proteins. Regions predicted to contain secondary structures are shown (42). Identical residues are shaded black, with similar residues shaded gray. Metal binding site 1 residues are indicated with circles, and site 2 residues are indicated with triangles, as identified from the crystal structure of *P. aeruginosa* Fur. Residues underlined highlight a motif thought to be involved in DNA binding. *Legionella pneumophila* (27), accession no. AAA19656.1; *Coxiella burnetii* (R. Seshadri and J. Samuel, unpublished results), accession no. AAK00303.1; *E. coli* (52), accession no. P06975; and *P. aeruginosa* (43), accession no. Q03456.

iron-limiting MAC agar, indicative of Fur-mediated repression in the reporter strain (see Materials and Methods). The recombinant plasmids all carried the same 2-kb ClaI fragment, and sequence analysis of one of these plasmids, pJIR2300, confirmed that a *fur* homologue was present. Southern hybridization analysis performed by using the ClaI insert in pJIR2300 as a probe showed that the insert hybridized to a 2-kb chromosomal ClaI fragment as expected (data not shown).

The *D. nodosus fur* gene was 420 bp and encoded a putative 16.2-kDa, 140-amino-acid cytoplasmic protein with a predicted isoelectric point of 5.42. No helix-turn-helix motif (14) was present. A putative sigma70 consensus binding site was located upstream of the translational start site, and there was a potential transcriptional-termination stem-loop structure ($\Delta G = -7.2$ kcal/mol) downstream of the translational stop codon.

The amino acid sequence of the Fur$_{Dn}$ protein had a high degree of similarity to many Fur proteins (Fig. 1), with the most closely related proteins including those from *Legionella pneumophila* (60% identity) and *E. coli* (61% identity). These proteins have discrete areas of similarity (Fig. 1), generally corresponding to the regions that are predicted to contain secondary structures (42). One of these regions, helix 4, contains a highly conserved motif Y55-F61 (numbering from *E. coli*) that is believed to be involved in DNA binding (21). A putative metal binding motif in the C-terminal end of the protein, H-H-X-H-X$_2$-C-X$_2$-C was only partially conserved,

with the Fur$_{Dn}$ protein having a threonine residue in place of the second cysteine. Located between the predicted second and third alpha helices there was also an inserted sequence (TSFD) that was unique to the Fur$_{Dn}$ protein (Fig. 1).

The genetic organization of the regions flanking the *fur* gene did not show a high degree of similarity to regions flanking other *fur* genes. Upstream of the *fur* gene in *D. nodosus* there was a gene encoding a homologue of the signal recognition receptor protein FtsY and a gene (*rpoH*) encoding a sigma32 homologue. In *Shewanella oneidensis* Fur was shown to regulate *rpoH* (58). Downstream of *fur* was a gene encoding a conserved hypothetical protein and a gene (*focA*) encoding a protein with similarity to formate or nitrite transporters.

The *D. nodosus* and *P. aeruginosa* Fur proteins have significant structural similarity. The recent elucidation of the crystal structure of the Fur$_{Pa}$ protein provided considerable insight into its mechanism of activation and DNA binding (42). The Fur$_{Dn}$ protein was modeled by using the structure of the Fur$_{Pa}$ protein as a reference point, and when the two structures were superimposed, they were virtually identical, which corresponded well with their predicted secondary structure similarities (data not shown). The only region that deviated significantly was the turn-loop region between helices two and three. This extended loop region contained the TSFD residues that were not present in any other Fur homologues. The structural effect of this extended loop was not profound, since it appeared
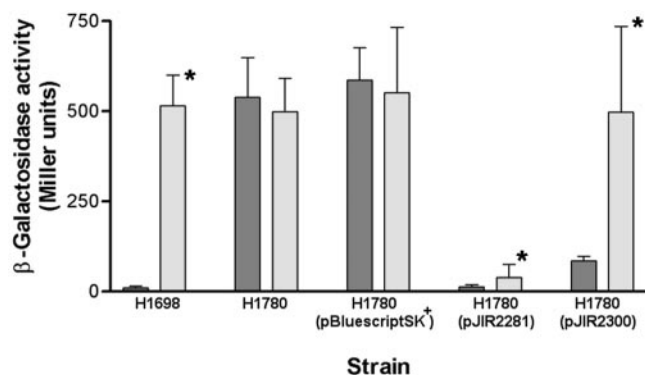
FIG. 2. Iron-dependent repression of a Fur-regulated promoter by the Fur_{Ec} and Fur_{Dn} proteins. The β-galactosidase activity of the *fiu-lacZ* fusion in *E. coli* is shown after growth under iron-replete (100 μM $FeCl_3$; dark gray) and iron-limiting (50 μM Desferal; light gray) conditions. Shown are the *fur*[+] (H1698) and *fur* mutant (H1780) reporter strains, H1780 containing the *E. coli* (pJIR2281), and *D. nodosus* (pJIR2300) *fur* genes on pBluescript SK[+] and a vector control. Each assay was performed at least four times in four independent experiments. Mean values are displayed with standard deviations as error bars. Asterisks indicate samples that were statistically different ($P <$ 0.05) when grown under iron-replete and iron-limiting conditions.

to bend back toward the third helix and created only a small increase to the surface of the protein in this area. This region was not on the DNA binding site of the Fur protein, nor was it close to the dimerization domain (16).

The Fur_{Pa} protein was crystallized in the presence of zinc, and subsequently two metal binding sites were identified. Binding site 1 represents the putative iron binding regulatory site and is coordinated by amino acids H86, D88, E107, and H124 (42). These residues are conserved among known Fur homologues (Fig. 1) but were not totally conserved in the Fur_{Dn} protein. In the Fur_{Dn} protein a change in charge was observed at position 107, where glutamate was replaced by threonine, and a significant change in charge occurred at position 124, with tyrosine in place of histidine. Site 2, coordinated by H32, E80, H89, and E100, was conserved in all of the Fur homologues, including the Fur_{Dn} protein(Fig. 1) (42).

The *D. nodosus fur* gene complements an *E. coli fur* mutant. The ability of H1780(pJIR2300) to form pink colonies on iron-replete MAC agar and red colonies on iron-limiting MAC agar suggested that fur_{Dn} was able to complement the fur_{Ec} mutation. To quantitate the extent of this complementation, the fur_{Dn} gene located on the high-copy-number vector pBluescript SK[+] was introduced into the reporter strain H1780, and β-galactosidase activity was assayed in the presence and absence of Desferal. Plasmid pJIR2281, which carried fur_{Ec} on pBluescript SK[+], was used as a multicopy-positive control. Strain H1698 was also assayed as it carries a functional *fur* gene, and in the presence of iron the resultant Fur protein represses the *lacZ* reporter gene. When iron is chelated with Desferal, apo-Fur is no longer able to bind to the *fiu* promoter, resulting in the production of β-galactosidase. The results showed that in the fur_{Dn} construct, β-galactosidase activity was highest when iron in the medium was limited, indicating iron-dependent regulation (Fig. 2). The control strains H1780 and H1780 (pBluescript SK[+]) showed high levels of β-galactosidase activ-

ity irrespective of the iron concentration. When the *fur* gene from *E. coli* was added, the presence of multiple copies of this gene in the reporter system resulted in β-galactosidase repression regardless of iron content, as observed elsewhere (37, 43, 56). In comparison to iron-limited cultures, the Fur_{Dn} protein was also able to significantly repress ($P <$ 0.05) the *fiu* promoter when supplemented with iron (Fig. 2). The degree of repression that was observed correlated well with the observation of pink colonies on MAC media. The ability of the fur_{Dn} gene to complement a fur_{Ec} mutant suggested that the Fur protein was likely to be functional in *D. nodosus*. In subsequent studies it was shown that a *fur* transcript could be detected in *D. nodosus* by RT-PCR and that *D. nodosus* strains encompassing various serogroups, as well as those representing virulent and benign isolates, all had the same 2-kb ClaI fragment that hybridized to a fur_{Dn} probe in Southern blot assays (data not shown).

Construction and analysis of a *D. nodosus fur* mutant. To determine the functional role of the *fur* gene in *D. nodosus*, a chromosomal *fur* mutant was constructed by allelic exchange. The suicide vector pJIR2417 was introduced into strain VCS1703A by natural transformation, and erythromycin-resistant colonies were selected. These resistant colonies were screened by using PCR, and two of these mutants (JIR3764 and JIR3765) were subjected to detailed analyses (see Materials and Methods) that confirmed they were derived from *D. nodosus* VCS1703A by double-crossover events that insertionally inactivated the *fur* gene (data not shown). All further studies were carried out on the *fur* mutant JIR3764.

To determine the potential biological effect of the *fur* mutation, the wild-type and mutant strains were grown under iron-replete and iron-limiting conditions. Although the growth rate of the *fur* mutant was the same as that of the wild-type in iron-replete medium, the use of the chelator 2,2′-dipyridyl to obtain iron-limiting conditions inhibited the already naturally poor growth of *D. nodosus* such that any differences were not able to be observed. In other bacteria, Fur has been shown to regulate the production of secreted proteins (16, 60). To see if the *fur* mutation had any effect on such proteins in *D. nodosus*, the wild-type and mutant strains were analyzed for their ability to secrete proteases, that is, to hydrolyze casein and elastase, as well as to carry out twitching motility, a property that is dependent on the production of type IV fimbriae. No difference was observed between the wild-type and mutant strains in these assays (data not shown).

Identification of Fur-regulated proteins by proteomic analysis. To determine the regulatory targets of Fur, a broader proteomic approach was employed, combining two-dimensional gel electrophoresis and MS. As Fur is known to regulate genes with a wide variety of functions, proteins from cell lysate, total membrane-enriched, and extracellular fractions were analyzed. The number of proteins that were observed over the three cellular fractions constituted a high proportion of the predicted *D. nodosus* proteome. The cell lysate had almost 1,000 protein spots, the membrane preparations had over 100 spots, and the secretome contained a surprisingly high number of at least 60 reproducible spots.

By comparing the proteome of the wild-type strain to that of the *fur* mutant JIR3764, we were able to observe and identify through MS several up-regulated and down-regulated proteins
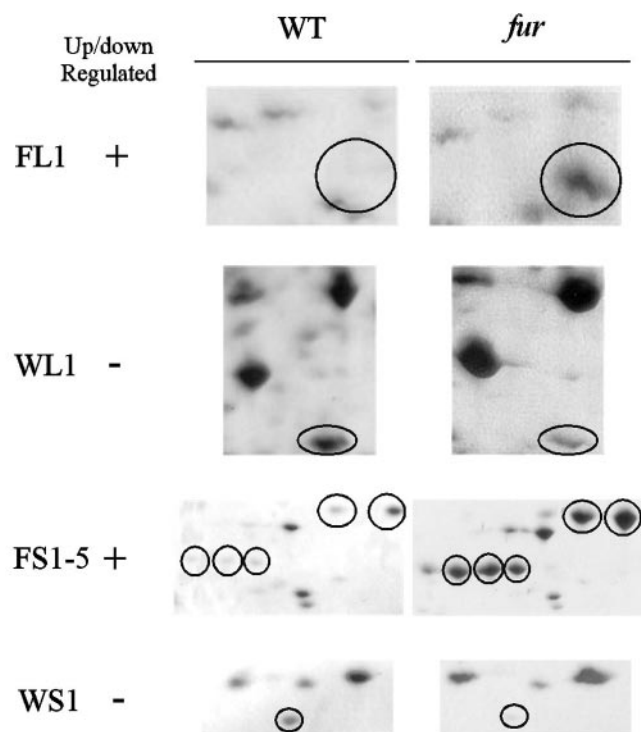
FIG. 3. Differentially expressed proteins in the *D. nodosus fur* mutant JIR3764. Shown are sections of representative gels of the wild-type strain VCS1703A and its *fur* mutant JIR3764. Spot numbers are designated according to the following key: F, up-regulated in *fur* mutant; W, down-regulated in *fur* mutant; L, identified in the cell lysate; and S, identified in the supernatant. Relevant bands are circled. Numbering for FS1 to FS5 is right to left, working down. Gels used for analytical purposes were loaded with 100, 45, and 30 μg of protein for cell lysate, total membrane, and secreted proteins, respectively. These values increased to 300, 100, and 200 μg, respectively, for successful micropreparative loadings.

in the *fur* mutant (Fig. 3 and Table 3). We were able to visualize other differences; however, from these spots we were either unable to obtain reproducible mass spectra or unable to identify a significant match to the database. These proteins were primarily from the membrane samples. QRT-PCR was subsequently used to verify that these regulatory effects occurred at the transcriptional level. Only those proteins for

which there was agreement between the proteomic and QRT-PCR data are reported here. There were four other proteins for which the proteomic and transcriptional profiles were at variance, presumably as a result of differences in posttranslational modification.

In the cell lysate the FL1 protein was identified as being up-regulated in the *fur* mutant. Significant matches were obtained to the YfeA proteins of *Pasteurella multocida* (39) and *Yersinia pestis* (5) and SitA from *S. enterica* serovar Typhimurium (67). YfeA or SitA represents the periplasmic binding protein of an ABC-type iron and manganese transport system (4, 5, 32) and is typically encoded within an operon, *yfeABCD* or *sitABCD*. YfeB is predicted to be an ATP-binding protein, and YfeC and YfeD are predicted to be integral membrane proteins (5). Levels of *yfeA* expression were higher in the *fur* mutant than in the wild type (Table 3).

One protein, WL1, was identified in the cell lysate as being down-regulated in the *fur* mutant (Fig. 3 and Table 3). This protein had a high degree of identity to the inducible version of ornithine decarboxylase, particularly that from *P. multocida* (64% identity) (39). QRT-PCR of the gene encoding the ornithine decarboxylase, *speF*, showed that levels of transcription were considerably reduced (Table 3).

BprV is one of the three extracellular serine proteases of *D. nodosus* and has a predicted pI of 9.5 (36). Since in other bacteria Fur has been shown to regulate the production of secreted proteins (16, 60), the secreted proteins from *D. nodosus* were analyzed over a pH 3 to 10 (not pH 4 to 7) range so that any potential effects on BprV could be detected, in addition to the two other acidic proteases. However, no differences in protein expression were detected in the pI 9.5 region. The secretome had five proteins, FS1 to FS5, clustered around 30 kDa, with pI values of 5.5, that were significantly up-regulated at the transcriptional level in the *fur* mutant (Fig. 3 and Table 3). MS showed that FS1 and FS2 were isoforms of the same protein previously detected in the cell lysate, FL1, which had similarity to the iron transport protein YfeA. All three of these proteins, FL1, FS1, and FS2, are encoded by the same ORF but feature various migratory patterns. The presence of this protein in the two cellular compartments is not surprising, considering that it is predicted to have a signal sequence. Proteins FS3, FS4, and FS5 were isoforms of another protein, which also had an N-terminal signal sequence.

TABLE 3. Differentially expressed proteins in the *D. nodosus fur* mutant JIR3764 and their expression ratios as determined by QRT-PCR

Spot no.[a]	Protein identification	Identity (%)	No. of peptides matched (% coverage)[c]	Relative gene expression[d]
FL1	YfeA, iron transport protein	64	10 (46)	1.44 ± 0.35
FS1	YfeA, iron transport protein	64	11 (60)	1.44 ± 0.35
FS2	YfeA, iron transport protein	64	11 (48)	1.44 ± 0.35
FS4	CbiK domain, ABC-type cobalt transport	29	15 (67)	4.28 ± 0.97
FS5	CbiK domain, ABC-type cobalt transport	29	19 (61)	4.28 ± 0.97
FS6	CbiK domain, ABC-type cobalt transport	29	11 (45)	4.28 ± 0.97
WL1	Ornithine decarboxylase[b]	64	16 (23)	0.38 ± 0.24
WS1	SodA, manganese superoxide dismutase	58	9 (48)	0.35 ± 0.02

[a] F, protein up regulated in *fur* mutant; W, protein present in wild-type, down-regulated in *fur* mutant; L, present in cell lysate; and S, present in secreted sample.
[b] Spot corresponded to a truncated form of a larger ORF.
[c] Number of experimentally derived mass peaks that match to the in silico derived peaks for that protein (degree of coverage of proteins conferred by the residues represented by matching peaks).
[d] Expressed as the ratio of the specific gene expression level in the *fur* mutant compared to wild-type normalized to the level of expression of the 16S rRNA gene.

These bands of protein spots had different isoelectric points, indicating that the protein undergoes some form of posttranslational processing or degradation. Although this protein only had moderate (29%) identity to a hypothetical protein from *Neisseria meningitidis*, it was identified as containing a conserved CbiK domain. CbiK is the periplasmic component of an ABC-type cobalt transport system (46). Consistent with the increased level of protein, expression of this gene, which we have designated as *cbiK,* showed a 4.28-fold increase in the *fur* mutant (Table 3).

A secreted protein, WS1, was observed to be down-regulated in the *fur* mutant compared to the wild type. WS1 had amino acid sequence similarity to a manganese superoxide dismutase (MnSOD), SodA, but did not have a signal sequence. The *D. nodosus* MnSOD homologue contained the signature pattern of iron and manganese containing SODs and had 58% identity to MnSOD from *Vibrio cholerae* (26). Analysis of the structural gene *sodA* by QRT-PCR showed that its expression was reduced nearly threefold in the *fur* mutant (Table 3).

An attempt was made to identify potential Fur boxes upstream of those genes that were negatively regulated by the Fur_{Dn} protein. Neither alignment of these upstream regions nor processing with motif finding programs revealed any significant similarities. Attempts at identifying putative Fur targets in the *D. nodosus* genome by using the *E. coli* consensus sequence did not reveal any obvious targets such as ferrocheletases or the ferrous iron transporters FeoA and FeoB that are encoded on the *D. nodosus* genome. The only significant match obtained was found upstream of the *cbiK* gene (encoding the FS3 to FS5 proteins), which was the gene shown to be highly regulated by the Fur_{Dn} protein at the transcriptional level (Table 3). It had 16 out of 19 bases conserved from the *E. coli* Fur box.

DISCUSSION

In the presence of iron Fur acts as a global transcriptional repressor, regulating a wide spectrum of genes that are involved in processes as diverse as iron metabolism, transport systems, oxidative stress, and virulence (16). We have identified the *D. nodosus fur* gene and used genetic and proteomic analysis to show that the Fur_{Dn} protein is functional and is involved in the regulation of genes involved in iron and manganese homeostasis and oxidative metabolism in *D. nodosus* (Table 3). These findings represent the first report of a functional regulatory protein in this important animal pathogen.

The classical role of Fur is to coordinate iron metabolism with iron availability by regulating iron uptake and acquisition systems (16). In this study two *D. nodosus* proteins were identified that potentially have similar functions. In other bacteria YfeA is the periplasmic binding protein component of an ABC transporter system that is involved in iron uptake. YfeA (or SitA) has been shown to be regulated by Fur in all of the organisms studied to date, including *Y. pestis* (5), *S. enterica* serovar Typhimurium (67), and *Shigella flexneri* (50). The proteins encoded by the *yfe* (or *sit*) operon also display similarity to manganese transporters, and subsequently the system has been shown to transport both iron and manganese (4, 32). The same operon structure observed with the *yfe* genes (*yfeABCD*)

was also observed in *D. nodosus* (data not shown). Detailed analysis of the *D. nodosus* genome sequence did not reveal any other iron- or manganese-specific ABC transport systems (unpublished data). Therefore, we postulate that YfeABCD may represent the primary transportation system by which iron or manganese enters the cell. The importance of this transport system is illustrated by reports that null mutants of the equivalent operon are attenuated for virulence in *Y. pestis* and *S. enterica* serovar Typhimurium (4, 30).

A protein (FS3, FS4, or FS5) containing a CbiK domain was also up-regulated in the *D. nodosus fur* mutant. CbiK is a cobalt chelatase that is involved in the anaerobic pathway of vitamin B_{12} (cobalamin) synthesis and in some species has been shown to invoke a human immune response (45, 49). Since CbiK from *S. enterica* serovar Typhimurium has been shown in vivo to act as a ferrochelatase by binding to Fe^{2+} (46), it is possible that in *D. nodosus* the CbiK homologue is a metal ion acquisition protein that may have a role in iron acquisition or iron and/or other metal ion homeostasis.

The *cbiK* gene was located only three genes downstream of the *fur* gene, after the putative formate or nitrate transport gene *focA*. No operon-like structure was evident. The only high-similarity match observed in searches based on the *E. coli* Fur box consensus sequence was *cbiK*. This Fur box conservation, in addition to the high level of Fur-dependent expression (Table 3), indicates that the Fur_{Dn} protein functions in a similar manner to the Fur_{Ec} protein.

The organization of the *D. nodosus fur* region was different from that of other characterized *fur* regions, although some conservation exists among members of the γ subclass of the *Proteobacteria* (37), to which *D. nodosus* belongs (13). The presence of an *rpoH* gene upstream of the *fur* gene was unique to *D. nodosus*, and although *rpoH* was not part of an operon with *fur* and there was no sigma32 consensus binding site in the *fur* promoter region, we cannot rule out the possibility that Fur may regulate *rpoH*, causing a discrete set of genes to be expressed, as observed in *S. oneidensis* (58).

In other bacteria, Fur links iron metabolism with oxidative metabolism and stress. Fur regulates SODs and is itself regulated by the oxidative stress regulators OxyR and SoxRS (23). This association with oxidative stress relates back to maintaining iron at a nontoxic level, as under aerobic conditions in the presence of iron-destructive hydroxy radicals can be produced via the Fenton reaction (2). This by-product of aerobic metabolism is not a concern for *D. nodosus* since it grows only under anaerobic conditions, which may explain why we did not observe a growth difference between the wild type and the *fur* mutant at different iron concentrations. A similar observation was made when *S. oneidensis* was grown anaerobically (58). Typically, a reduced growth rate is observed under aerobic iron-replete conditions, as seen in *Y. pestis* (53), *Neisseria gonorrhoeae* (57), and *Staphylococcus aureus* (29). This difference is less profound or is absent under microaerophilic or anaerobic conditions due to reduced radical toxicity and the increased solubility of iron, as demonstrated in *P. aeruginosa* (25), *Helicobacter pylori* (6) and *S. oneidensis* (58).

SODs catalyze the dismutation of oxygen radicals to hydrogen peroxide. In *E. coli*, Fur acts as a transcriptional repressor of the MnSOD gene, *sodA*, and as an indirect positive regulator of the iron-dependent superoxide dismutase (FeSOD)

gene, *sodB* (16, 38). In *D. nodosus*, we observed a novel Fur regulatory effect whereby MnSOD expression in the *fur* mutant was reduced, resulting from a 2.8-fold reduction in *sodA* transcription (Table 3). This scenario is analogous to the situation with *sodB* in *E. coli*. Our analysis of the *D. nodosus* genome sequence did not reveal the presence of a FeSOD gene. Therefore, we postulate that in *D. nodosus* MnSOD may represent a functional homologue of FeSOD and may be regulated by both iron and manganese levels. In *E. coli*, the regulation of *sodB* is undertaken by a small RNA molecule, RyhB, that is regulated by Fur (38). This molecule is, in turn, regulated by the RNA chaperone Hfq (62). A RyhB homologue was not present in *D. nodosus*; however, a Hfq homologue with 62% identity to *E. coli* Hfq was present. This analysis does not preclude the presence of a functional RyhB homologue in *D. nodosus*, as observed in *P. aeruginosa* (65), since similarity between small RNA molecules of distantly related organisms is not high (64, 65). As *D. nodosus* is an aerotolerant anaerobe, the role of SOD may only be apparent at the site of infection, before an anaerobic environment is established, or as protection from phagocytic cells (48). We did not observe any alteration in growth of the *fur* mutant after 4 h of exposure to air (data not shown), although at this point in time nothing is known about the mechanisms of aerotolerance in *D. nodosus*. The identification of MnSOD in the culture supernatant is unusual, considering that it is a cytoplasmic protein lacking a signal peptide. Its presence is likely to be the result of cellular leakage and extracellular stability, as previously reported in other organisms (28, 61).

In other bacteria the effects of *fur* mutations on virulence have been mixed. A *fur* mutant of *Campylobacter jejuni* was restricted in its ability to colonize chickens (41), and in *S. aureus* and *Listeria monocytogenes* attenuation was observed in murine models (29, 47). In *S. enterica* serovar Typhimurium mutation of *fur* gave various results, depending on the route of delivery (19). By contrast, no effect on virulence was observed in *P. multocida* (8) or avian strains of *E. coli* (68). The presence of the *fur* gene in both virulent and benign strains of *D. nodosus* would suggest that it is not specifically required for virulent footrot. However, Fur may still regulate genes in virulent strains that have an effect on virulence or may regulate genes that are common to virulent and benign strains but, when differentially expressed, have an effect on virulence. Further studies are required to determine if the *D. nodosus* homologues of the Fur, YfeA, MnSOD, or CbiK proteins are involved in virulence.

The Fur$_{Dn}$ protein was able to significantly repress the *fiu* promoter in the reporter strain in an iron-dependent manner but not to the same degree as the Fur$_{Ec}$ protein (Fig. 2). The difference in levels of repression may be due to the heterologous nature of the system and fundamental differences in Fur binding preferences. This potential binding site preference may also reflect the scarcity of identifiable Fur boxes in the *D. nodosus* genome. Since Fur proteins are capable of binding other metal ions (11), the Fur$_{Dn}$ protein may also bind other metals instead of, or in addition to, iron. Although the sequence for metal binding site 1 is not absolutely conserved in the Fur$_{Dn}$ protein, the complementation data indicate that sufficient structural and functional similarity is present to enable the Fur$_{Dn}$ protein to complement the *E. coli* system and to

be responsive to iron levels, indicating that the activity of the Fur$_{Dn}$ protein is associated with iron. The absence of an *E. coli*-like Fur binding consensus sequence has been observed elsewhere (18, 41). With other Fur proteins the degree of repression observed in this *fiu* reporter system is usually analogous to that of *E. coli* (56, 66). Only in *P. aeruginosa* has a degree of repression similar to the results of this study been observed (43). Both the Fur$_{Dn}$ protein (61%) and the Fur$_{Pa}$ protein (58%) have similar identity to the Fur$_{Ec}$ protein. The only major structural difference between the Fur$_{Pa}$ and Fur$_{Dn}$ proteins is in the loop region between the second and third helices, where the Fur$_{Dn}$ protein has the unique motif TSFD. The second cysteine in the C-terminal H-H-X-H-X$_2$-C-X$_2$-C metal binding site is changed to a threonine residue in both the *P. aeruginosa* and *D. nodosus* proteins, which may explain the altered repression observed in *E. coli*. These observations are in agreement with previous studies (11) which showed that Fur activity in *E. coli* was compromised when the same cysteine residue was changed to serine, with the derepression/repression ratio being comparable to that seen in this study and with the Fur$_{Pa}$ protein(43).

In summary, we have identified and then characterized through insertional inactivation the first transcriptional regulator to be functionally analyzed in *D. nodosus*. The Fur$_{Dn}$ protein had extensive structural homology to the Fur$_{Pa}$ protein and was able to complement an *E. coli fur* mutant. Comparison of the proteomes of the wild-type strain VCS1703A and its *fur* mutant JIR3764 identified several differentially expressed genes and implicated Fur as a regulator of iron and oxidative metabolism in this anaerobe.

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