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Ferric Uptake Regulator Fur Control of Putative Iron Acquisition Systems in *Clostridium difficile*

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ABSTRACT

***Clostridium difficile* is an anaerobic, Gram-positive, spore-forming opportunistic pathogen and is the most common cause of hospital-acquired infectious diarrhea. Although iron acquisition in the host is a key to survival of bacterial pathogens, high levels of intracellular iron can increase oxidative damage. Therefore, expression of iron acquisition mechanisms is tightly controlled by transcriptional regulators. We identified a *C. difficile* homologue of the master bacterial iron regulator Fur. Using targetron mutagenesis, we generated a *fur* insertion mutant of *C. difficile*. To identify the genes regulated by Fur in *C. difficile*, we used microarray analysis to compare transcriptional differences between the *fur* mutant and the wild type when grown in high-iron medium. The *fur* mutant had increased expression of greater than 70 transcriptional units. Using quantitative reverse transcriptase PCR (qRT-PCR), we analyzed several of the Fur-regulated genes identified by the microarray and verified that they are both iron and Fur regulated in *C. difficile*. Among those Fur- and iron-repressed genes were *C. difficile* genes encoding 7 putative cation transport systems of different classes. We found that Fur was able to bind the DNA upstream of three Fur-repressed genes in electrophoretic mobility shift assays. We also demonstrate that expression of Fur-regulated putative iron acquisition systems was increased during *C. difficile* infection using the hamster model. Our data suggest that *C. difficile* expresses multiple iron transport mechanisms in response iron depletion *in vitro* and *in vivo*.**

IMPORTANCE

***Clostridium difficile* is the most common cause of hospital-acquired infectious diarrhea and has been recently classified as an "urgent" antibiotic resistance threat by the CDC. To survive and cause disease, most bacterial pathogens must acquire the essential enzymatic cofactor iron. While import of adequate iron is essential for most bacterial growth, excess intracellular iron can lead to extensive oxidative damage. Thus, bacteria must regulate iron import to maintain iron homeostasis. We demonstrate here that *C. difficile* regulates expression of several putative iron acquisition systems using the transcriptional regulator Fur. These import mechanisms are induced under iron-limiting conditions *in vitro* and during *C. difficile* infection of the host. This suggests that during a *C. difficile* infection, iron availability is limited *in vivo*.**

Almost all living organisms require iron as a cofactor for essential metabolic chemistry (1). Although iron is one of the most abundant of Earth's elements, ferric iron has very limited solubility in aqueous, nonacidic, or oxygenated environments (1). Ferric iron is most often found as iron oxides or hydroxides, which cannot be used by most organisms. Competition over bioavailable iron is fierce both among bacteria in complex microbial communities and between bacterial pathogens and their eukaryotic hosts (2).

Organisms have evolved various mechanisms of iron transport to obtain this essential cofactor. Many bacteria produce low-molecular-weight, high-affinity iron chelators known as siderophores (3). Specific ABC transporters translocate iron-bound siderophores into cells. The siderophores can be made by the organism itself or coopted from other prokaryotic or eukaryotic neighbors (3). Under anaerobic or low-pH conditions, ferrous iron predominates over the ferric form. The solubility of ferrous iron is orders of magnitude higher than that of ferric iron, and thus it can be directly transported into cells as a free metal. Bacteria are known to transport free ferrous iron using G-protein-like ferrous permeases (4).

Although iron acquisition is crucial for survival, high levels of intracellular iron can react with hydrogen peroxide to form reactive hydroxyl radicals. These reactive radicals can damage DNA as well as iron-containing enzymes (5, 6). Thus, maintenance of appropriate intracellular iron levels in bacteria is an important task. Many Gram-positive and Gram-negative bacteria utilize a transcriptional regulator called Fur (ferric uptake regulator) to control intracellular iron homeostasis (7–9). In these organisms, Fur is known to regulate expression of multiple proteins involved in survival under iron-limiting conditions, including iron acquisition systems. In most known examples, Fur binds intracellular iron and binds DNA as a homodimer (7, 10). Most commonly, the Fe-Fur dimer binds to DNA near the promoter regions of genes in the Fur regulon, thus blocking transcription (7, 11). Expression of

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TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Genotype or description	Reference[a] or source
Clostridium difficile strains		
JIR8094	Spontaneous erythromycin-sensitive derivative of 630	12
TCD90	JIR8094 fur_{300}::*ltrB*::*ermB*	
TCD91	JIR8094 fur_{300}::*ltrB*::*ermB*/pRPF185	
TCD93	JIR8094 fur_{300}::*ltrB*::*ermB*/pTHE884	
Escherichia coli strains		
Omnimax-2 T1R	F′ [$proAB^+$ $lacI^q$ *lacZ*ΔM15 Tn*10*(Tet^r) Δ(*ccdAB*)] *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 endA1 recA1 supE44 thi-1 gyrA96 relA1 tonA panD*	Invitrogen
HB101/pRK24	F^- *mcrB mrr hsdS20*(r_B^- m_B^-) *recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20*(Smr) *glnV44* λ^- pRK24	15
Plasmids		
pBL100	P_{fac} *ltrB*::*ermBRAM ltrA cat repA orfB oriT*	13
pRPF185	P_{tet}-*gus colE1 bla cat repA orfB oriT aad9*	17
pTHE627	P_{fac} $ltrB_{fur}$::*ermBRAM ltrA cat repA*	
pTHE884	P_{fur}-$fur_{C.\ difficile}$-*gus colE1 bla cat repA orfB oriT aad9*	

[a] This study unless otherwise noted.

Fur-repressed genes is lower in the presence of iron and higher under iron-depleted conditions.

In this study, we generated a *Clostridium difficile fur* insertion mutant. This mutant was used to identify iron-repressed genes whose expression was regulated by Fur in an iron-dependent manner. Expression of several Fur-regulated *C. difficile* genes was induced in infected hamsters, suggesting that iron is limiting during a *C. difficile* infection.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and primers. The bacterial strains and plasmids used in this study are described in Table 1. The *C. difficile* strains are isogenic with the erythromycin-sensitive strain JIR8094, a derivative of the sequenced clinical isolate 630 (12). *C. difficile* was grown in or on TY medium (0.4% tryptone, 0.5% yeast extract) at 37°C in an atmosphere of 10% hydrogen, 5% CO_2, and 85% nitrogen in an anaerobic chamber (Coy Laboratory Products). To determine low-iron medium conditions, we performed MIC experiments with dipyridyl. We found that 250 μM dipyridyl inhibited growth of wild-type *C. difficile*, while growth was not inhibited by up to 500 μM $FeCl_3$. Low-iron TY medium contained dipyridyl (final concentration, 100 μM; Sigma), and high-iron TY medium was supplemented with $FeCl_3$ (final concentration, 250 μM; RPI Corp.).

C. difficile strains were grown on TY agar (2%) plates containing thiamphenicol (Thi) (10 μg/ml), erythromycin (Erm) (5 μg/ml), or kanamycin (Kan) (50 μg/ml) as needed. Optical densities (ODs) of bacterial cultures were measured using a WPA spectrophotometer (CO800 cell density meter).

Escherichia coli strains were grown in or on LB medium (1% tryptone, 0.5% yeast extract, 0.5% sodium chloride) at 37°C with ampicillin (Amp) (50 μg/ml) or chloramphenicol (Cam) (10 μg/ml) as needed. The primers used in this work are listed in Table S1 in the supplemental material. All primers were synthesized by IDT DNA Inc. (Coralville, IA).

Plasmid and bacterial strain construction. Plasmid cloning was performed in the *E. coli* strain Omnimax-2 T1R (Invitrogen). To construct a *fur* mutant, we retargeted the intron Ll.LtrB from plasmid pBL100 (13). The primers were designed using the Clostron algorithm (14). The intron was amplified from a targetron template (Sigma) using the TE2125, TE2126, and TE2127 primers (see Table S1 in the supplemental material). This insert was digested with HindIII and BsrGI and cloned into the pBL100 plasmid digested with the same restriction enzymes. The resulting plasmid, pTHE627, was transformed into the conjugation donor HB101/pRK24 (15) to move the retargeted plasmid into *C. difficile* to generate the *fur* intron insertion mutant as previously described (16). The insertion of the intron into *fur* was confirmed by PCR using oligomers TE2721 and TE2280 (see Table S1 in the supplemental material).

For complementation of the *fur* mutant, we cloned the *C. difficile fur* open reading frame and 200 bp of the 5′ upstream region by PCR amplifying DNA, using TE2596 and TE2597 (see Table S1 in the supplemental material). The resulting PCR product was cloned into the pRPF185 plasmid (17) which had been digested with the NheI and SacI restriction enzymes using Gibson Assembly (NEB). The P-*fur* construct (pTHE884) was maintained in Omnimax or HB101/pRK24 cells grown at 30°C prior to introduction into *C. difficile*.

Bioinformatic analysis. Gene sequences from bacterial genomes were obtained from the BioCyc website. The multiple-sequence alignment of Fur proteins from *C. difficile*, *Bacillus subtilis*, and *E. coli* was generated using the default settings of Clustal Omega (18). The consensus Fur-binding sequence for *C. difficile* was determined by analyzing ~250 bp upstream and ~50 bp downstream of the predicted translational start site for the first gene each of the 8 Fur-repressed operons encoding transporters using MEME, a motif-based sequence analysis tool (19). The MEME software was used with settings to identify consensus regions between 16 and 22 bp.

Isolation of *C. difficile* nucleic acids. *C. difficile* chromosomal DNA was purified as previously described (16). *C. difficile* RNA was isolated from bacteria grown in TY medium. For each sample, a single colony of *C. difficile* was inoculated in TY medium and grown overnight in high-iron-containing TY medium. Overnight cultures were washed in phosphate-buffered saline (PBS) and diluted 1:25 in low- or high-iron-containing TY medium grown to an OD and 600 nm (OD_{600}) of 0.6 to 0.9. Cells were fixed by adding equal volumes of acetone-ethanol (1:1) to cells and incubated at −80°C for at least 30 min. Fixed cells were pelleted by centrifugation, washed 3 times with 0.75 ml of diethyl pyrocarbonate (DEPC)-treated water, and then resuspended in 0.6 ml of buffer RLT (Qiagen) with β-mercaptoethanol (final concentration, 10%; Sigma). Cells were disrupted by sonication (10 pulses of setting 3 for 1 s; Branson Sonifier 150). RNA was extracted using the RNeasy RNA isolation kit (Qiagen). Contaminating DNA was removed using the Turbo DNA-free kit (Ambion). Samples were tested for DNA contamination by PCR amplification (Thermo *Taq* polymerase; NEB) using primers TE485 and TE486 (see Table S1 in the supplemental material).

Microarray analysis. Samples used for microarray analysis were obtained from the wild type (JIR8094) and *fur* mutant (TCD90) grown in high-iron-containing TY medium to an OD of 0.8. The cells were fixed with an acetone-ethanol mixture (at a 1:1 ratio). RNA was isolated from these fixed cells using Trizol reagent (Invitrogen) as previously described (16, 20). The resulting RNA was treated with DNase I (Turbo DNA-free kit; Ambion) and purified with RNeasy spin columns (Qiagen). The resulting RNA was processed by the University of Iowa Carver Center for Genomics (Iowa City, IA) with custom *C. difficile* Roche Nimblegen microarrays (20). RNA from three independent biological replicates grown on different days was used. Each of the biological replicates was tested in technical duplicate on the microarray slides. Data analysis workflow was performed with the Partek Genomics Suite (Partek Inc.).

qRT-PCR. To generate cDNA from RNA samples, we used Superscript II (Invitrogen) or Moloney murine leukemia virus (MMuLV) reverse transcriptase (RT) (NEB) according to the manufacturer's protocols. The resulting reverse transcription reaction mixtures were diluted 1:5 in DEPC-treated water. For each quantitative RT-PCR (qRT-PCR), 5 μl of sample was added to 10 μl of Power Sybr green master mix (Applied Biosystems) and 5 μl gene-specific primers (2 × 2.5 μM). The list of primers used to quantitate cDNA levels of different samples is provided in Table S1 in the supplemental material. Experiments were performed in technical triplicate on three biologically independent replicates. Data were normalized to RNA levels of the *C. difficile* housekeeping gene *rpoB* (for *in vitro* experiments) or the *C. difficile*-specific *mldA* gene (for *in vivo* experiments), which was chosen due to its presence almost exclusively in *C. difficile* (21). Thus, *mldA* primers would not cross-react with commensal bacteria.

EMSA. We attempted to express the Fur protein in *E. coli* cloning and protein expression strains using several different plasmid constructs. Most of the clones that we were able to obtain had point mutations in Fur which likely rendered the protein inactive or truncated. We were able to obtain and express N-terminally His-tagged Fur protein but found this protein to be inactive. Subsequent cleavage by the AcTEV protease did not generate sufficient quantities of active Fur protein for electrophoretic mobility shift assays (EMSAs). Thus, we synthesized the Fur protein using *in vitro* transcription and translation. For a *fur*-encoding template, we introduced the T7 promoter using 2 consecutive PCRs. The first *Taq* polymerase reaction (NEB) used oligomers CDEP3202 and CDEP3203 (see Table S1 in the supplemental material). This product was gel purified (Fermentas) and then used as the template for a subsequent PCR with oligomers CDEP3221 and CDEP3203 (see Table S1 in the supplemental material). This final product was used as the template in a PURExpress *in vitro* protein synthesis reaction (NEB) according to the manufacturer's instructions.

For the EMSAs, we PCR amplified putative promoter regions upstream of the *cd1477* (TE2723 and TEQ130; 310 bp), *cd2992* (TE2247 and TE2248; 305 bp), and *fur* (TE2281 and TE2722; 375 bp) genes. These DNA products were gel purified (Fermentas), and 132 ng of DNA for each probe was labeled using T4 polynucleotide kinase (NEB) with [γ-^{32}P]ATP (PerkinElmer). Each radioactively labeled DNA probe was cleaned using a mini-spin column (Fermentas) and used in EMSA reactions.

For each EMSA reaction, 1 μl of DNA probe was incubated with 1× EMSA buffer (20 mM Tris HCl [pH 8], 50 mM KCl, 5% glycerol, 1 mM dithiothreitol [DTT], 1 mM $MgCl_2$) with salmon sperm DNA (1 μg/ml; Invitrogen) and bovine serum albumin (BSA) (1 μg/ml; NEB) for 5 min at room temperature. One microliter of Fur *in vitro* synthesis reaction mixture or, as a negative control, 1 μl of mock *in vitro* synthesis reaction mixture (PURExpress *in vitro* protein synthesis with no template) was added to EMSA reaction mixtures and allowed to incubate for 30 min at room temperature under normal atmospheric oxygen conditions. Each reaction mixture was loaded on a vertical 4% acrylamide–bis-Tris-acetate-EDTA (TAE) gel and electrophoresed at 83 V for 3 h at 4°C. The gel was then immobilized on filter paper, wrapped in plastic wrap, and exposed to a phosphor screen for 1 to 2 h. The phosphor screen was then imaged using a Typhoon 8610 variable-mode imager.

***C. difficile* gene expression during hamster model of infection.** *C. difficile* spores used for infections were prepared as previously described (22). Briefly, the *C. difficile* wild type (0.3 ml of overnight culture) was spread on TY plates and incubated for 3 days at 37°C in an anaerobic chamber. The cells were resuspended in phosphate-buffered saline (PBS), and the vegetative cells were killed by heat inactivation at 65°C aerobically for 30 min. Spore preparations were washed extensively in PBS and stored at 4°C prior to use. Spore counts were determined by plating serial dilutions on TY plates containing 0.1% taurocholate.

Four adult Syrian gold hamsters (~90 to 120 g; Harlan Sprague-Dawley, Inc., Indianapolis, IN) were inoculated orally with clindamycin (Sigma; 30 mg/kg) 5 days prior to infection (23). Hamsters were inoculated with 10,000 spores of wild-type *C. difficile*. Hamsters were monitored twice daily for signs of severe morbidity and euthanized prior to death. After euthanization, 1-cm sections of infected ceca were removed and individually fixed in 1 ml Trizol (Invitrogen). After storage at −20°C, samples were thawed and homogenized. RNA was isolated from these samples as previously described (16). The animal experiments performed in this study were approved by the University of Iowa Institutional Animal Care and Use Committee.

Microarray data accession numbers. The microarray design (GPL20243) and data (GSE69218) have been deposited at the Gene Expression Omnibus (GEO) database at NIH.

RESULTS

Construction of *C. difficile* Fur mutant. The transcriptional regulator Fur plays a major role in controlling iron homeostasis in most Gram-positive and Gram-negative bacteria (7). To study regulation of iron acquisition, we constructed a *fur* mutant of *C. difficile*. The *fur* gene was identified from the *C. difficile* 630 genome using the online bioinformatics tool BioCyc. The *C. difficile* Fur homologue is 62% and 70% similar to the Fur proteins of *E. coli* and *Bacillus subtilis*, respectively. Alignment of the Fur proteins from *C. difficile*, *E. coli*, and *B. subtilis* shows that the Zn^{2+}-binding region, the Fe^{2+}-binding region necessary for homodimerization, and the DNA-binding regions were conserved between the Fur proteins (Fig. 1A).

To construct a *fur* mutant of *C. difficile*, we used the targetron method to create an erythromycin resistance-marked insertion mutation as previously described (14, 16, 24). The insertion of the intron into the *fur* gene was confirmed by PCR (Fig. 1B). We then tested the ability of the *C. difficile fur* mutant to grow in low- and high-iron-containing media. The *C. difficile fur* mutant demonstrated no growth defect compared to the wild-type parent under the high- or low-iron conditions. However, during late stationary phase, the optical density of the *fur* mutant decreased compared to that of the wild-type parent, suggesting that a significant number of the *fur* mutant cells lysed at some time during stationary phase (see Fig. S1 in the supplemental material).

Identification of *C. difficile* genes regulated by Fur by microarray analysis. To identify Fur-regulated genes, we isolated RNA from either the wild type or the *fur* mutant grown in the presence of iron. In bacteria where Fur controls gene expression in response to iron levels, Fur binds its target promoters in the presence of iron. Fur-repressed genes have lower expression in the wild type but are derepressed in the absence of Fur even in the presence of iron. Using microarray analysis, we identified greater than 70 *C. difficile* putative transcriptional units (single genes or putative operons) which had higher expression in the *fur* mutant (Fur repressed) than in the wild type (2.5-fold change cutoff and *P*

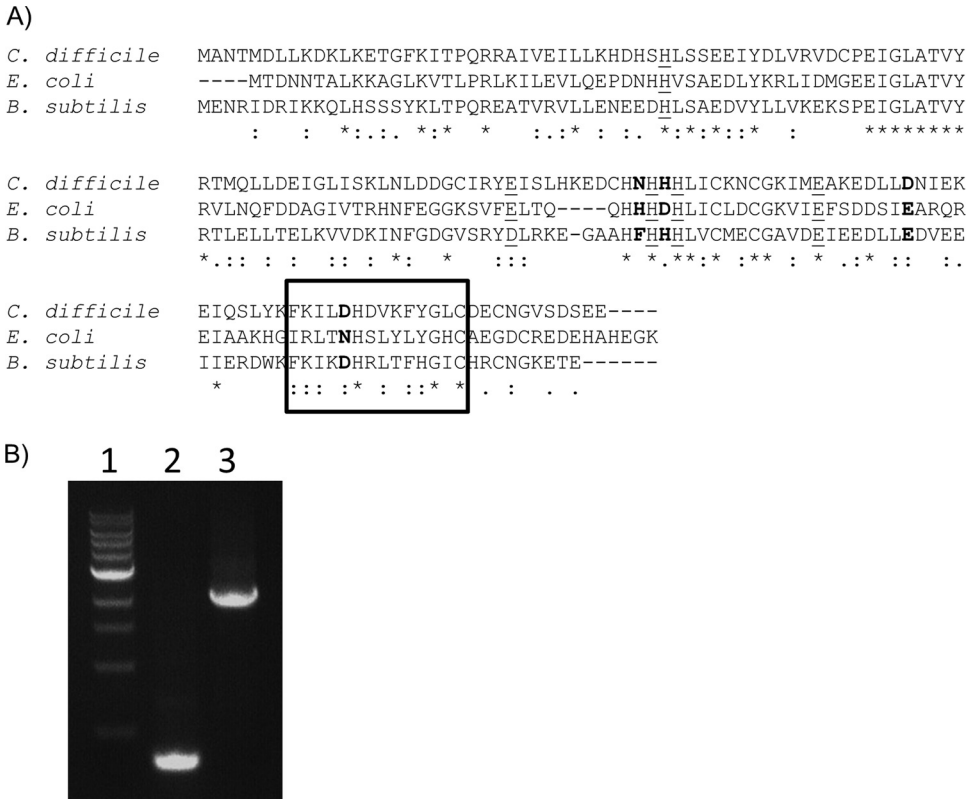

FIG 1 *C. difficile* ferric uptake regulator Fur. (A) Alignment of Fur proteins from *C. difficile*, *B. subtilis*, and *E. coli*. The alignment was made with Clustal Omega (18) using default parameters and the following sequence accession numbers: *E. coli*, NP_415209.1; *B. subtilis*, NP_390233.2; and *C. difficile*, YP_001087781.1. The amino acids required for Zn^{2+} binding are underlined. The amino acids required for Fe^{2+} binding are in bold. The boxed region is the dimerization domain. (B) PCR Confirmation of wild type and *fur*::*ltrB*::*erm* mutant using primers TE2721 and TE2280, homologous to the 5′ and 3′ ends of the *fur* gene, respectively. Lane 1, 1-kb ladder; lane 2, wild-type chromosomal DNA; lane 3, *fur* mutant chromosomal DNA.

value limit of $\leq 10^{-8}$). Additionally, the majority of the genes carried on the ϕC630-1 and ϕC630-2 prophages and the CTn*4* and CTn*6* conjugative transposons were also Fur repressed (Table 2). We found that 44 transcriptional units exhibited 2.5- to 11-fold-lower expression in the *fur* mutant than in the wild type, suggesting that these genes are directly or indirectly induced in the presence of Fur (Table 2).

Iron regulation of Fur-regulated genes. To verify the effect of Fur on expression of genes identified in our microarray analysis, we performed quantitative reverse transcriptase PCR (qRT-PCR) on several genes of interest. As in the microarray analysis, we isolated RNA from the *fur* mutant and wild-type cells grown in the presence of high iron concentrations. The RNA from each sample was converted to cDNA and quantified using qRT-PCR. For 14 of the 15 genes which were identified as Fur repressed by microarray analysis, mRNA levels were significantly higher in the *fur* mutant than in the wild type (Table 3). Furthermore, the levels of repression in the qRT-PCR experiments were comparable to the levels from the microarray analysis.

To further examine the iron regulation of our genes of interest, we compared RNA levels of the Fur-repressed genes from wild-type cells grown under low- or high-iron conditions. Fourteen genes which were found to be Fur repressed were also iron repressed to an extent similar to their Fur regulation (Table 3). Interestingly, two genes (*cd3118* and *cd1889*) were regulated by Fur but not repressed in the presence of iron. Similar Fur-repressed iron-independent regulation has been observed with the AmiF foramidase in *Helicobacter pylori* (25), and this suggests that regulation of *cd3118* and *cd1889* may be more complicated.

We identified 8 Fur-repressed putative transport systems (Table 2). Of these, 3 (*cd1647-cd1650*, *cd2992-cd2989*, *cd2874-cd2878*, and *cd1891-cd1892*) were homologous to ABC transporters. The *cd1647-cd1650* and *cd2874-cd2878* operons are highly similar to the genes encoding the Fpi (catecholate) and Fhu (hydroxamate) siderophore transport systems, respectively. Two of the Fur- and iron-repressed operons (*feo1* and *feo3*) were homologous to the Feo ferrous transport system. The remaining two operons (*cd0592*-1 and *cd1087*) encoded proteins similar to P-type cation transporters and the low-affinity zinc transporter ZupT, respectively.

To further demonstrate that the *fur* mutation was responsible for the regulation of these putative transport systems, we complemented the *fur* mutant with Fur expressed from a low-copy-number plasmid (26, 27). When grown in the presence of iron, the *fur* mutant containing an empty vector exhibited between 120- and 350-fold higher expression of the *feo1*, *fpi*, and *zupT* operons than did the wild type (Fig. 2). We observed no significant differences in *feo1*, *fpi*, and *zupT* RNA levels between the wild type and the *fur* mutant carrying the Fur-expressing plasmid (Fig. 2). This suggests that the regulation defect of the *fur* mutant can be complemented in *trans* by expressing *fur* from a plasmid.

Fur also regulated expression of other genes which are not

TABLE 2 Genes regulated in the *fur* mutant

Category and gene name[a]	Function[b]	Fold change[c]	*P* value[d]
Fur-repressed genes			
cd1647-cd1650 (*fpi*)	Catecholate siderophore ABC transport	216 to 730	<1.03E−15
cd1485	Hypothetical protein	449	4.16E−17
cd0592-cd0591	Putative P-type cation transport	136 to 351	<8.49E−14
cd1087 (*zupT*)	*zupT* transporter	341	9.29E−14
cd2499	Hypothetical protein	269	7.92E−17
cd1088	Hypothetical protein	142	6.84E−16
cd1477-cd1480 (*feo1*)	*feo1* ferrous iron transport operon	36.6 to 162	<4.68E−15
cd1119	Putative lipoprotein	50.6	3.63E−15
cd2992-2989	ABC transporter	23.6 to 38.7	<1.03E−14
cd3118	Hypothetical protein	36.3	1.63E−15
cd1999 (*fldX*)	Flavodoxin	31.1	1.71E−11
cd1089-cd1090	Two-component system	25.4 to 30.3	<7.82E−16
cd2874-cd2878 (*fhu*)	Hydroxamate siderophore ABC transporter	4.40 to 26.1	<1.13E−11
cd1887-8 (*csfU-rsiU*)	ECF sigma factor CsfU operon	4.75 to 23.1	<4.23E−10
cd0593	Hypothetical protein	7.57	1.58E−16
cd1085-cd1086	Hypothetical protein, putative peptidase	6.41 to 7.27	<2.92E−12
cd3091 (*treA*)	Trehalose-6-phosphate hydrolyase	5.94	1.00E−12
cd0762-8 (*gut-srl*)	Glucitol/sorbitol-specific PTS	2.37 to 5.62	<7.04E−08
cd1287 (*fur*)	Ferric uptake regulator	5.36	1.63E−10
cd1120-2 (*dhaB*)	Glycerol dehydratase	2.15 to 5.23	<9.10E−12
cd2216	Hypothetical protein	4.51	1.43E−12
cd0740	Putative aminotransferase	4.09	4.75E−12
cd2351 (*grdB*)	Glycine reductase complex B γ subunit	3.85	2.20E−13
cd0594-cd0595	Hypothetical proteins	3.35 to 3.84	<2.64E−13
cd2987-2988	Two-component system	3.30 to 3.80	<7.34E−12
cd3273-cd3274	*feo3* ferrous iron transport operon	2.92 to 3.49	<2.62E−13
cd1565-6 (*ilvC-ilvB*)	Ketol-acid reductoisomerase	2.98 to 3.44	<1.20E−10
cd1819	Hypothetical protein	3.34	1.79E−10
cd1745A(*feoA4*)	*feoA4* ferrous iron transport protein A	3.30	1.41E−09
cd0596-8 (*cotJB1-cotJC1*)	Putative spore coat proteins	2.80 to 3.29	<1.60E−10
cd0797	Hydroxymethylglutaryl-CoA lyase	3.8	2.44E−12
cd2418-6 (*srlA-srlE*)	Glucitol/sorbitol-specific PTS	2.79 to 3.18	<9.06E−12
cd1820 (*ade*)	Adenine deaminase	3.09	1.52E−13
cd1564	Hypothetical protein	2.98	1.42E−08
cd0798	Hypothetical protein	2.88	7.59E−12
cd0995-6 (*serA*)	D-3-Phosphoglycerase dehydrogenase	2.39 to 2.77	<5.59E−11
cd2000 (*ispD*)	Major intracellular serine protease	2.73	3.33E−09
cd2324-3 (*gatD*)	Galactitol-1-phosphate 5-dehydrogenase	2.15 to 2.58	<2.55E−08
ϕC630-1	Phage proteins	2.15 to 4.33	<2.55E−08
ϕC630-2	Phage proteins	2.70 to 4.78	<4.60E−10
CTn4	Conjugative transposon genes	3.62 to 4.02	<2.20E−07
CTn6	Conjugative transposon genes	2.90 to 3.73	<1.12E−06
Fur-induced genes			
cd2214-5	Putative regulatory proteins	−6.83 to −11.1	<1.86E−11
cd2822-20	Hypothetical proteins	−5.49 to −9.58	<1.07E−14
cd2169-8	Hydroxylamine reductase	−3.13 to −7.93	6.70E−12
cd2003 (*effD*)	Efflux pump	−7.01	1.85E−09
cd2241-39 (*nanE-nanA*)	*N*-Acetyl-neuraminate, mannosamine degradation	−5.21 to −6.43	<3.26E−12
cd2276	Putative Na:solute symporter	−6.30	8.05E−14
cd2738-2737	Cytosine permease, C-N hydrolase	−5.86 to −5.57	<1.13E−14
cd0902	Putative cation efflux protein	−5.38	4.54E−14
cd3096-3095	6-Phospho-beta-glucosidase	−4.49 to −5.33	<4.92E−11
cd3097	PTS, IIABC component	−4.98	2.27E−11
cd0899 (*dinB*)	DNA polymerase IV	−4.66	1.77E−14
cd2593	ABC transporter, ATP-binding component	−4.55	1.47E−12
cd0332 (*bclA1*)	Putative exosporium glycoprotein	−4.49	4.91E−11
cd2230 (*nirC*)	Putative nitrite transporter	−4.43	4.07E−12
cd2629 (*spoIVA*)	Stage IV sporulation protein A	−4.23	2.93E−15
cd2055	Hypothetical protein	−4.01	1.49E−09
cd0587-0588	Hypothetical proteins	−3.55 to −4.00	<9.89E−12

(Continued on following page)

TABLE 2 (Continued)

Category and gene name[a]	Function[b]	Fold change[c]	*P* value[d]
cd2479	Hypothetical protein	−3.88	1.58E−11
cd1753-1755	Putative ABC transporter	−2.05 to −3.71	<2.11E−08
cd0126 (*spoIIID*)	Stage III sporulation protein D	−3.68	1.27E−09
cd3264	Putative membrane protein	−3.55	2.02E−09
cd2097	Putative membrane protein	−3.50	2.82E−10
cd0892 (*cspA*)	Cold shock protein	−3.50	2.74E−09
cd3417-3416	Putative ABC transporter	−2.02 to −3.49	<2.08E−08
cd1752	Putative transcriptional regulator	−3.43	1.51E−11
cd0581-2	Transcriptional regulator, PEP kinase	−2.83 to −3.33	<2.40E−10
cd1993	Putative carboxylase	−3.20	9.49E−10
cd2566-7	PTS, IIA IIB component	−2.12 to −3.09	<9.49E−11
cd2206 (*aldH*)	Aldehyde dehydrogenase	−2.97	1.07E−12
cd2121	Hypothetical protein	−2.96	2.07E−07
cd0773 (*spoVAC*)	Stage V sporulation AC	−2.93	1.49E−08
cd3515	Pilin	−2.90	8.95E−09
cd0739	Putative exported protein	−2.89	1.91E−11
cd0488 (*sugE*)	Quaternary ammonium resistance protein	−2.80	1.14E−06
cd1192-4 (*spoIIIAA,AB,AC*)	Stage III sporulation protein AA, AB, AC	−2.59 to −2.76	<2.08E−07
cd2231-3 (*asrABC*)	Anaerobic sulfite reductase	−2.19 to −2.76	<1.67E−08
cd0106 (*cwlD*)	Germination specific *N*-acetylmuramoyl–L-alanine amidase	−2.73	1.69E−09
cd3520	Putative cation efflux protein	−2.69	3.27E−13
cd0589-590	Hypothetical proteins	−2.21 to −2.66	<3.43E−10
cd0670	Regulatory protein	−2.65	5.85E−11
cd1063B-1063C	Hypothetical proteins	−2.56 to −2.63	<2.08E−05
cd1291 (*dacF*)	D-Alanyl-D-alanine carboxypeptidase	−2.63	2.28E−11
cd2310 (*cspD*)	Cold shock protein	−2.60	8.71E−08
cd2749A-50 (*agrBD*)	Auotinducer peptide, regulator	−2.51 to −2.57	<2.41E−08

[a] From GenBank.
[b] Putative functions as determined by current annotation of the *C. difficile* genome. PTS, phosphotransferase system; CoA, coenzyme A; PEP, phosphoenolpyruvate.
[c] The fold changes listed are averages from three biological replicates, with each done in technical replicates. Fold changes signify expression that is increased in the *fur* mutant compared to the wild-type *C. difficile* when both strains were grown in high-iron-containing medium. For genes in a putative operon, the range of fold change is reported.
[d] The *P* values listed are averages from three biological replicates, with each done in technical replicates. For genes in a putative operon, the highest *P* value is reported.

predicted to transport ions. We found that expression of 2 putative two-component systems operons was induced by iron depletion and was Fur repressed. These regulators (*cd1089-1090* and *cd2987-2988*) (Table 3) may be involved in a regulatory cascade in which Fur indirectly controls expression of genes in its regulon. Additionally, expression of one flavodoxin gene, *fldX*, was strongly repressed by Fur and by high iron, while one ferredoxin gene (*cd0627A*) had lower expression in the *fur* mutant or under low-iron conditions.

Fur binding to Fur-regulated promoters. We used the bioinformatics software MEME (19) to identify a putative Fur-binding site in the promoter regions of the Fur-regulated genes identified as encoding putative transporters. We compared 250 bp upstream through 50 bp downstream of the translational start sites of 8 highly Fur-repressed *C. difficile* genes (Fig. 3A). We were able to identify a highly homologous 18-bp region in these sequences (Fig. 3A and B). The putative Fur-binding consensus sequence of *C. difficile* is similar to the consensus Fur-binding sites from the 19-bp consensus sequence identified in *B. subtilis* (28).

We performed electrophoretic mobility shift assays (EMSAs) to demonstrate the binding of Fur protein to putative Fur-regulated promoters. In these assays, Fur protein was synthesized using *in vitro* transcription and translation. When the promoter regions of the *cd1477* (*feo1* operon), *fur*, and *cd2992* genes were incubated with the product of the Fur *in vitro* transcription-translation reaction, we observed a shift of each of these promoter fragments (Fig. 3C). Importantly, we did not observe a shift the mobility of the DNA when incubation was with the mock transcription-translation reaction mixture (Fig. 3C). In addition, we did not observe a Fur-induced shift when we tested a probe including the *feoA2* open reading frame, which is not predicted to contain a Fur-binding site and was not regulated by Fur (Fig. 3C). Taken together, these findings suggest that Fur likely acts to directly repress expression of at least some of these genes.

Expression of Fur-regulated genes during *C. difficile* infection of hamsters. Eukaryotic hosts are thought to sequester available iron to limit the growth of bacterial pathogens. Under these iron-limiting conditions, it is advantageous for the bacteria to induce expression of iron uptake mechanisms. To determine whether expression of Fur-regulated genes was increased *in vivo*, we measured the expression levels of Fur-regulated putative cation acquisition genes during wild-type *C. difficile* infection of the hamster cecum. Hamsters were infected with wild-type *C. difficile* spores and exhibited at least 2 days of diarrhea before animals were sacrificed. The most distal and most proximal 1-cm cecal sections of each infected cecum (4 animals) were removed and immediately fixed in Trizol for RNA isolation. In these qRT-PCR experiments, we compared RNA levels of our genes of interest to the levels of *mldA*. Previous work has shown that the *C. difficile*-specific cell division gene *mldA* must be expressed at a low, constitutive level *in vitro* and *in vivo* (21).

We measured expression of 7 Fur-regulated putative ion trans-

TABLE 3 Fur- and iron-dependent regulation of *C. difficile* genes

Gene name[a]	Fur repression in qRT-PCR[b]	Iron repression in qRT-PCR[c]	Primers[d]
cd1647 (*yclO*)	9,600	920	TEQ081, TEQ082
cd1485	930	180	TEQ089, TEQ090
cd0591	81.3	1,200	TEQ085, TEQ086
cd1087 (*zupT*)	679	327	TEQ083, TEQ084
cd2499	270	189	TEQ091, TEQ092
cd1477	118	38.5	TEQ129, TEQ130
cd1489 (*feoB1*)	41.1	92	TEQ061, TEQ062
cd2992	71.7	95.7	TEQ077, TEQ078
cd3118	12.3	3.54	TEQ093, TEQ094
cd1999 (*fldX*)	154	162	TEQ087, TEQ088
cd2878 (*fhuD*)	800	867	TEQ065, TEQ066
cd1887 (*csfU*)	0.46	0.455	TEQ005, TEQ006
cd1889	4.58	0.792	TEQ079, TEQ080
cd1287 (*fur*)	17.0	3.62	TEQ099, TEQ100
cd1517 (*feoB2*)	0.500	1.17	TEQ057, TEQ058
cd3273 (*feoA3*)	4.51	5.61	TEQ059, TEQ060
cd1745A (*feoA4*)	12.7	3.55	TEQ097, TEQ098
cd0627A (ferredoxin)	−4.9	ND[e]	TEQ105, TEQ106
cd2214	−2.90	ND	TEQ109, TEQ110

[a] From GenBank.
[b] Fur repression is the level of gene expression in the *fur* mutant divided by that in the wild-type *C. difficile* strain when both strains were grown in high-iron-containing medium. The reported values are the arithmetic averages from three biological replicates, with each done in technical replicates.
[c] Iron repression is the level of gene expression in wild-type *C. difficile* grown in low-iron-containing medium divided by that in wild-type *C. difficile* grown in high-iron-containing medium. The reported values are the arithmetic averages from three biological replicates, with each done in technical replicates.
[d] Sequences for the DNA primers used in qRT-PCRs are listed in Table S1 in the supplemental material.
[e] ND, not determined.

port systems during *C. difficile* infection. Each cation transport operon demonstrated higher expression in the infected cecum than when the wild type was grown under iron-replete conditions, but to different extents (Fig. 4). The *cd0591* and *fpi* operons exhibited the highest induction under both low-iron and *in vivo* conditions (Fig. 4). Expression of the *feo1*, *zupT*, *cd2992*, and *fhu* operons also showed substantial *in vivo* induction, although the overall RNA levels of *fhu* were considerably lower than those of other Fur-regulated genes (Fig. 4).

Interestingly, expression profiles of the 3 *C. difficile* FeoB permeases were significantly different. Although *feo2* expression was not responsive to iron levels (Table 2; Fig. 4), it may be induced ~10-fold during infection (Fig. 4). This would suggest that *feo2* expression may be regulated in a Fur- and iron-independent manner. Our data suggest that *feo3* expression is significantly less repressed by Fur (Table 2) and iron (Table 2; Fig. 4) than is *feo1* expression. In accordance with these observations, there appears to be less induction of *feo3* during *in vivo* infection as well (Fig. 4). In comparison, the *feo1* operon is highly induced *in vivo* and under low iron conditions (Fig. 4). Taken together, our data suggest that Fur-regulated, iron-regulated genes are induced during *C. difficile* infection of the hamster cecum, thus suggesting that iron is limited during a *C. difficile* infection.

DISCUSSION

We have been investigating the role of the transcriptional repressor Fur in *C. difficile*. Here we have identified and confirmed the *C. difficile* Fur regulon. We have demonstrated Fur and iron regulation of several classes of ion transporters in *C. difficile in vitro* and *in vivo*.

It is well established that many bacteria encode ABC transporters which import iron-bound siderophores (29). Siderophores can be classified into 3 major groups: hydroxamates, catecholates, or mixed-ligand siderophores (30). We have found that *C. difficile* encodes a putative Fhu (ferric hydroxamate uptake) ABC transporter system which is expressed under iron-limiting conditions and is repressed by Fur. Fhu transporters have been shown to function in iron acquisition in many Gram-positive as well as Gram-negative bacteria (31–34). *C. difficile* also encodes a system homologous to the catecholate siderophore petrobactin ABC transporter Fpi/Ycl from *B. subtilis* (35). Like that of *fhu*, *fpi* expression is both iron and Fur repressed. In *B. subtilis*, Fpi imports petrobactin, the primary siderophore produced by *Bacillus anthracis* strains but not synthesized by *B. subtilis* itself (35).

In addition to these putative siderophore ABC transporters, we have identified two other Fur- and iron-regulated ABC transporters (CD2992-2989 and CD1891-1892). At this time, the substrates for CD2992-2989 and CD1891-1892 have not yet been determined. So far, bioinformatics has not provided any clues as to the identity of the substrate for CD1891-2. However, bioinformatic analysis indicates that *C. difficile* CD2992-2989 is structurally similar to the ABC transporter systems SsuCBA, which likely imports aliphatic sulfonates. Unlike *ssuCBA* which is not regulated by Fur or iron (data not shown), we found that *cd2992-2989* is in the Fur regulon. To our knowledge, ABC transporters of sulfonated siderophores have not been characterized. However, members of the *Marinobacter* genus have been shown to synthesize a sulfonated siderophore (36, 37). It is possible that CD2992-2989 has specificity for a sulfonated derivative of a siderophore.

Often siderophore biosynthesis genes are also regulated by Fur (3). However, we have not found evidence for siderophore biosynthesis genes repressed by Fur, nor has bioinformatics revealed any obvious homologues to known siderophore biosynthesis genes in the *C. difficile* genome. It is possible that *C. difficile* synthesizes its own siderophores via an uncharacterized mechanism. It is also possible that *C. difficile* coopts siderophores produced by neighboring bacteria such as the resident microflora *C. difficile*

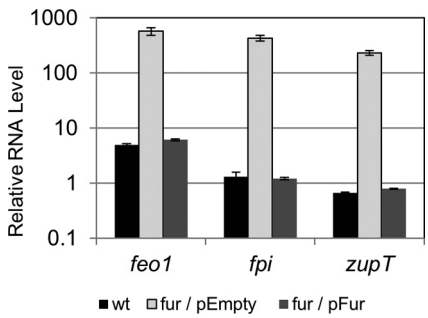

FIG 2 Complementation of Fur-regulated genes in *C. difficile*. The wild type (wt), the *fur* mutant containing empty vector (*fur*/pEmpty), and the *fur* mutant containing the P_{fur}-*fur*$^{+}$ plasmid (*fur*/pFur) were grown to mid-log phase (OD, 0.8) in TY medium with a high iron concentration. The mRNA levels of *feo1*, *fpi*, and *zupT* were normalized to the level of *rpoB* transcript in each sample using the primers listed in Table S1 in the supplemental material. The data are graphed as the arithmetic mean and standard deviation of three biological replicates.

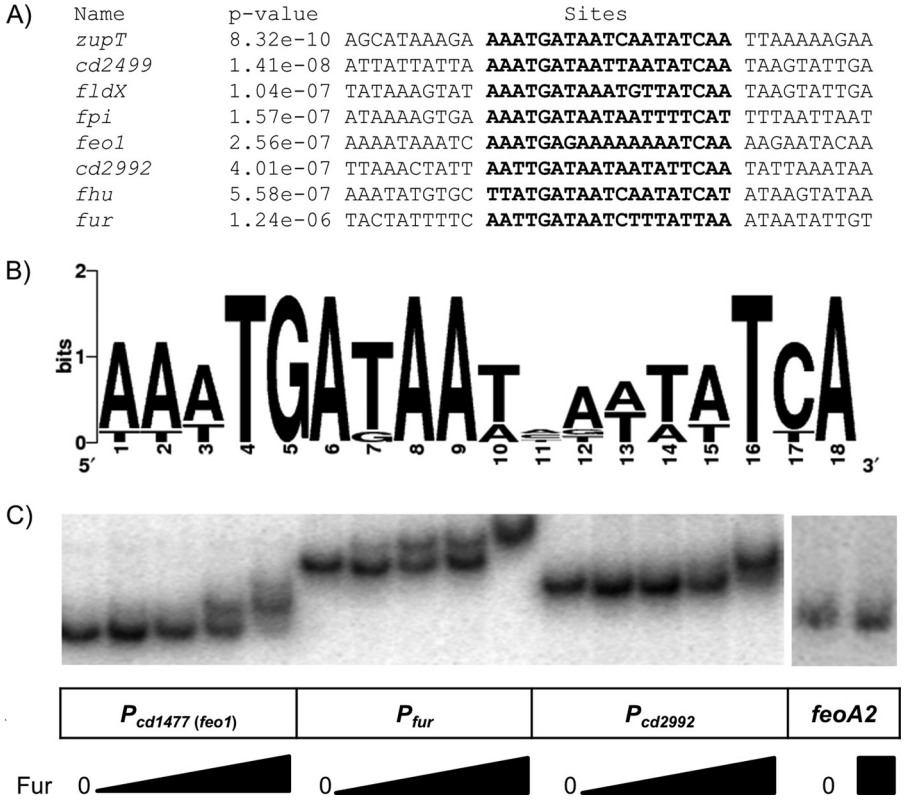

FIG 3 Fur binding of iron-regulated promoters. (A) Alignment of the Fur-binding regions of iron-regulated promoters. The consensus sequence was determined using MEME software with settings to identify consensus regions between 16 and 22 bp and using ~250 bp upstream and 50 bp downstream of the predicted start of translation for each of the Fur-repressed genes. In bold is the predicted Fur-binding site. (B) Consensus sequence logo of the *C. difficile* Fur-binding region. (C) Fur electrophoretic mobility shift assays. A [γ-^{32}P]ATP-labeled DNA probe was incubated with either the *in vitro* transcription-translation reaction mixture of a mock control (0) or increasing amounts of *in vitro* transcription-translation Fur protein for 30 min. Protein was diluted 1:16, 1:8, 1:4, and 1:2 in 1× EMSA binding buffer prior to addition to the DNA probe.

encounters during infection. Under anaerobic conditions, iron is found in the ferrous form. As a strict anaerobe, *C. difficile* may be more dependent upon transport of free ferrous iron rather than ferric iron.

In many bacteria, transport of ferrous iron is dependent upon a class of transporters known as Feo transporters (4). In the canonical example, these transporters require a membrane protein, FeoB, and a cytoplasmic protein, FeoA. Some bacteria encode single Feo transporter systems, while others harbor multiple Feo paralogues. For example, *Porphyromonas gingivalis* encodes 2 Feo transport systems, one of which transports ferrous iron while the other imports Mn^{2+} (38). The genomes of all sequenced *C. difficile* strains available include genes encoding 3 paralogous Feo transport systems, which we have termed *feo1*, *feo2*, and *feo3*. In our

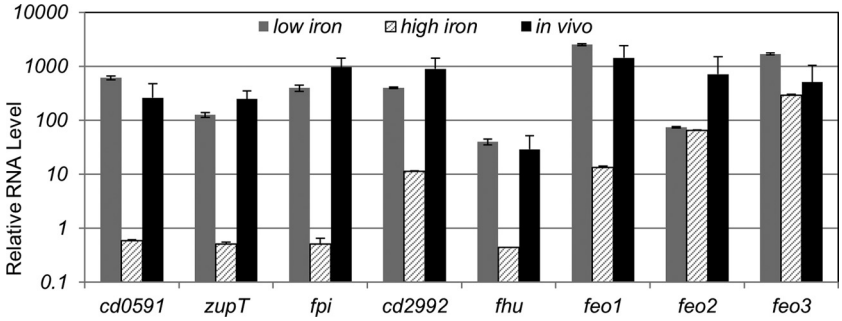

FIG 4 Expression of putative cation transporter genes during infection of hamster model. To determine the expression of Fur-repressed genes during a *C. difficile* infection, hamsters were infected with 10,000 wild-type spores 5 days after receiving a single dose of clindamycin. Two days after the initial onset of diarrhea, infected hamsters were sacrificed. The ceca of infected hamsters were removed, and RNA was extracted from the cecal samples. The mRNA levels of *cd0591*, *zupT*, *fpi*, *cd2992*, *fhu*, *feo1*, *feo2*, and *feo3* were normalized to the level of *mldA* transcript in each sample using the primers listed in Table S1 in the supplemental material. The mRNA levels were compared to those in wild-type cells grown in high-iron (250 μM $FeCl_3$) TY and low-iron (100 μM dipyridyl) TY. Experiments were performed in technical and biological triplicate. The data are graphed as the arithmetic mean with standard deviation.

work, we have found that expression of the Feo2 system is not repressed by Fur or iron levels. It is possible that this Feo system transports a cation other than iron, as is the case for *P. gingivalis*.

The remaining 2 Feo systems of *C. difficile* are Fur and iron regulated. Of these two Feo systems, the Feo1 system is more highly expressed and more strongly Fur regulated. Unlike the other two *C. difficile feo* operons, the *feo1* operon includes 2 additional small open reading frames flanking the annotated genes encoding the cytoplasmic protein FeoA and the membrane-bound FeoB permease. In *Yersinia pestis*, FeoC, encoded by the third gene in the *feo* operon, is not required for Feo transport activity but is involved in regulation of the system (39). In *V. cholerae*, the FeoC protein does not affect expression of the *feo* operon but is essential for Feo transport, although its role in Feo transport is not known (40). In *C. difficile*, the role of these small proteins in the Feo1 system is not known.

In addition to the Feo systems, we also identified two putative ion transporters belonging to different families of divalent cations transporters whose expression was repressed in the presence of Fur and iron. CD0591 is homologous to the P-type family of cation transporters (41). CD1087 is similar to ZupT, a broad-specificity divalent metal cation transporter (42). Members of both of these transporter families can be found in prokaryotes and eukaryotes (42–44). Homologues of CD0591 and CD1087 in other bacteria have been shown to transport multiple divalent cations, including Mn^{2+}, Zn^{2+}, Cd^{2+}, and Fe^{2+} (43, 45, 46). While several of these cation transporters are capable of importing ferrous iron, they have higher affinity for other divalent cations (46). It is possible that CD0591 and CD1087 are important for transporting multiple metal ions.

Although *C. difficile* and *B. subtilis* both utilize Fur as a regulator of iron homeostasis, many other ion-sensing transcriptional regulators found in *B. subtilis* (MntR, Zur, and PerR) are not identifiable by homology in *C. difficile* (47, 48). For example unlike *B. subtilis*, *C. difficile* does not encode a zinc-sensing Zur or a manganese-responsive MntR homologue. Presently, it is unclear how *C. difficile* may sense levels of zinc or manganese and control their homeostasis. However, expression of ion transporters can be affected by the levels of metal ions which they do not transport. For example, *E. coli* MntH imports Mn^{2+} but is repressed by both the iron-sensing Fur and the manganese-responsive MntR regulators (49). Manganese can serve as a substitute for iron in many metalloenzymes under iron-depleted conditions (50). Thus, one hypothesis is that expression of *cd0591* and *cd1087* may be repressed by Fur because the CD0591 and CD1087 transporters may be importing Mn^{2+} as an alternative enzyme cofactor when iron is scarce.

In addition to iron transport, we demonstrate Fur regulation of ferredoxin and flavodoxin. Ferredoxin and flavodoxin are isofunctional electron transfer proteins involved in numerous metabolic reactions (51). Ferredoxins require iron-sulfur clusters to coordinate electron transfer, while flavodoxins use flavin mononucleotide (FMN)/flavins and do not require iron. Interestingly, *C. difficile* encodes 4 putative ferredoxins and 6 potential flavodoxins. We have found that the flavodoxin gene *fldX* is induced under iron-limiting conditions, while *cd0627A* is repressed. This regulation is dependent upon Fur, suggesting that it may play a role in the balance of ferredoxin-flavodoxin expression.

We also found that expression of several mobile elements was higher in the *fur* mutant than in wild-type *C. difficile*. While it is possible that Fur directly represses expression of key regulators of these elements, Fur repression of mobile element induction may be more indirect. In the absence of Fur, *C. difficile* presumably imports higher levels of iron, which can catalyze oxidative radical production from the Fenton reaction, ultimately leading to increased DNA damage. Many phages and conjugative transposons use DNA damage as a signal for induction of mobile element excision from the chromosome (52). Phage induction can lead to lysis of the *fur* mutant and may account for the drop in optical density that we observed in the *fur* mutant during stationary growth phase. As an obligate anaerobe, *C. difficile* is extremely sensitive to oxidative stress. Iron potentiates the damaging effects of oxidative stress. Thus, Fur may play an important role in obtaining an adequate iron level for iron-dependent metabolism while avoiding excessive intracellular iron levels that could cause damage to DNA.

Recently it has been appreciated that Fur not only represses but can directly act as a transcriptional activator in *Helicobacter pylori* (53), *Campylobacter jejuni* (54), and *Neisseria meningitidis* (8). Our microarray analyses suggest that a class of *C. difficile* genes have lower expression in the *fur* mutant. These genes may be directly activated by Fur or may be indirectly controlled by a Fur-dependent regulator. Future experiments on *C. difficile* Fur binding to Fur-activated promoters may distinguish the role of Fur in activation of *C. difficile* gene expression.

Having determined iron repression of several putative ion transport systems *in vitro*, we investigated their expression during a *C. difficile* infection. We tested several Fur-regulated genes for *in vivo* expression in the hamster model of *C. difficile* infection, which mimics the pathology of a *C. difficile* infection in humans (23). In this work, we demonstrate substantial increases in expression of all the Fur-regulated genes that we tested. Our data strongly suggest that *C. difficile* induces expression of the Fur regulon during infection of the hamster cecum. They further suggest that the hamster cecum may be an iron-limiting environment. A previous study of *in vivo C. difficile* gene transcription suggested that none of the Fur-regulated genes were induced during the first 38 h of colonization of the gnotobiotic mouse (55). This would imply that early colonization of the gnotobiotic mouse model iron is not limiting. This difference in iron levels between the gnotobiotic mouse and hamster models of infection may be due to differences in the host sequestration of iron or the lack of resident microflora competing for available iron in the gnotobiotic mouse. Our data suggest that Fur plays an important role in regulating a large class of proteins induced during hamster infection. Dysregulation of Fur-regulated genes may have significant impact on *C. difficile* survival during infection.

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REFERENCES

1. **Andrews SC, Robinson AK, Rodríguez-Quiñones F.** 2003. Bacterial iron homeostasis. FEMS Microbiol Rev **27**:215–237. http://dx.doi.org/10.1016/S0168-6445(03)00055-X.
2. **Skaar EP.** 2010. The battle for iron between bacterial pathogens and their

vertebrate hosts. PLoS Pathog **6:**e1000949. http://dx.doi.org/10.1371/journal.ppat.1000949.
3. **Miethke M, Marahiel MA.** 2007. Siderophore-based iron acquisition and pathogen control. Microbiol Mol Biol Rev **71:**413–451. http://dx.doi.org/10.1128/MMBR.00012-07.
4. **Cartron ML, Maddocks S, Gillingham P, Craven CJ, Andrews SC.** 2006. Feo–transport of ferrous iron into bacteria. Biometals **19:**143–157. http://dx.doi.org/10.1007/s10534-006-0003-2.
5. **Imlay JA, Linn S.** 1988. DNA damage and oxygen radical toxicity. Science **240:**1302–1309. http://dx.doi.org/10.1126/science.3287616.
6. **Imlay JA.** 2003. Pathways of oxidative damage. Annu Rev Microbiol **57:**395–418. http://dx.doi.org/10.1146/annurev.micro.57.030502.090938.
7. **Troxell B, Hassan HM.** 2013. Transcriptional regulation by ferric uptake regulator (Fur) in pathogenic bacteria. Front Cell Infect Microbiol **3:**59. http://dx.doi.org/10.3389/fcimb.2013.00059.
8. **Grifantini R, Sebastian S, Frigimelica E, Draghi M, Bartolini E, Muzzi A, Rappuoli R, Grandi G, Genco CA.** 2003. Identification of iron-activated and -repressed Fur-dependent genes by transcriptome analysis of Neisseria meningitidis group B. Proc Natl Acad Sci U S A **100:**9542–9547. http://dx.doi.org/10.1073/pnas.1033001100.
9. **Porcheron G, Garénaux A, Proulx J, Sabri M, Dozois CM.** 2013. Iron, copper, zinc, and manganese transport and regulation in pathogenic Enterobacteria: correlations between strains, site of infection and the relative importance of the different metal transport systems for virulence. Front Cell Infect Microbiol **3:**90. http://dx.doi.org/10.3389/fcimb.2013.00090.
10. **Stojiljkovic I, Hantke K.** 1995. Functional domains of the Escherichia coli ferric uptake regulator protein (Fur). Mol Gen Genet **247:**199–205. http://dx.doi.org/10.1007/BF00705650.
11. **Baichoo N, Wang T, Ye R, Helmann JD.** 2002. Global analysis of the Bacillus subtilis fur regulon and the iron starvation stimulon. Mol Microbiol **45:**1613–1629. http://dx.doi.org/10.1046/j.1365-2958.2002.03113.x.
12. **O'Connor JR, Lyras D, Farrow KA, Adams V, Powell DR, Hinds J, Cheung JK, Rood JI.** 2006. Construction and analysis of chromosomal Clostridium difficile mutants. Mol Microbiol **61:**1335–1351. http://dx.doi.org/10.1111/j.1365-2958.2006.05315.x.
13. **Bouillaut L, Self WT, Sonenshein AL.** 2013. Proline-dependent regulation of Clostridium difficile Stickland metabolism. J Bacteriol **195:**844–854. http://dx.doi.org/10.1128/JB.01492-12.
14. **Heap JT, Kuehne Sa, Ehsaan M, Cartman ST, Cooksley CM, Scott JC, Minton NP.** 2010. The ClosTron: mutagenesis in Clostridium refined and streamlined. J Microbiol Methods **80:**49–55. http://dx.doi.org/10.1016/j.mimet.2009.10.018.
15. **Trieu-Cuot P, Carlier C, Poyart-salmeron C, Courvalin P.** 1991. Shuttle vectors containing a multiple cloning site and a lacZa gene for conjugal transfer of DNA from Escherichia coli to Gram-positive bacteria. Gene **102:**99–104. http://dx.doi.org/10.1016/0378-1119(91)90546-N.
16. **Ho TD, Ellermeier CD.** 2011. PrsW is required for colonization, resistance to antimicrobial peptides, and expression of extracytoplasmic function σ factors in Clostridium difficile. Infect Immun **79:**3229–3238. http://dx.doi.org/10.1128/IAI.00019-11.
17. **Fagan RP, Fairweather NF.** 2011. Clostridium difficile has two parallel and essential Sec secretion systems. J Biol Chem **286:**27483–27493. http://dx.doi.org/10.1074/jbc.M111.263889.
18. **Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Söding J, Thompson JD, Higgins DG.** 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol Syst Biol **7:**539. http://dx.doi.org/10.1038/msb.2011.75.
19. **Bailey TL, Boden M, Buske Fa, Frith M, Grant CE, Clementi L, Ren J, Li WW, Noble WS.** 2009. MEME SUITE: tools for motif discovery and searching. Nucleic Acids Res **37:**W202–W208. http://dx.doi.org/10.1093/nar/gkp335.
20. **Ho TD, Williams KB, Chen Y, Helm RF, Popham DL, Ellermeier CD.** 2014. Clostridium difficile extracytoplasmic function σ factor σV regulates lysozyme resistance and is necessary for pathogenesis in the hamster model of infection. Infect Immun **82:**2345–2355. http://dx.doi.org/10.1128/IAI.01483-13.
21. **Ransom EM, Williams KB, Weiss DS, Ellermeier CD.** 2014. Identification and characterization of a gene cluster required for proper rod shape, cell division, and pathogenesis in Clostridium difficile. J Bacteriol **196:**2290–2300. http://dx.doi.org/10.1128/JB.00038-14.
22. **Sambol SP, Tang JK, Merrigan MM, Johnson S, Gerding DN.** 2001. Infection of hamsters with epidemiologically important strains of Clostridium difficile. J Infect Dis **183:**1760–1766. http://dx.doi.org/10.1086/320736.
23. **Goulding D, Thompson H, Emerson J, Fairweather NF, Dougan G, Douce GR.** 2009. Distinctive profiles of infection and pathology in hamsters infected with Clostridium difficile strains 630 and B1. Infect Immun **77:**5478–5485. http://dx.doi.org/10.1128/IAI.00551-09.
24. **Heap JT, Pennington OJ, Cartman ST, Carter GP, Minton NP.** 2007. The ClosTron: a universal gene knock-out system for the genus Clostridium. J Microbiol Methods **70:**452–464. http://dx.doi.org/10.1016/j.mimet.2007.05.021.
25. **Van Vliet AHM, Stoof J, Poppelaars SW, Bereswill S, Homuth G, Kist M, Kuipers EJ, Kusters JG.** 2003. Differential regulation of amidase- and formamidase-mediated ammonia production by the Helicobacter pylori fur repressor. J Biol Chem **278:**9052–9057. http://dx.doi.org/10.1074/jbc.M207542200.
26. **Heap JT, Pennington OJ, Cartman ST, Minton NP.** 2009. A modular system for Clostridium shuttle plasmids. J Microbiol Methods **78:**79–85. http://dx.doi.org/10.1016/j.mimet.2009.05.004.
27. **Ransom EM, Ellermeier CD, Weiss DS.** 2015. Use of mCherry red fluorescent protein for studies of protein localization and gene expression in Clostridium difficile. Appl Environ Microbiol **81:**1652–1660. http://dx.doi.org/10.1128/AEM.03446-14.
28. **Baichoo N, Helmann JD.** 2002. Recognition of DNA by Fur: A reinterpretation of the Fur box consensus sequence. J Bacteriol **184:**5826–5832. http://dx.doi.org/10.1128/JB.184.21.5826-5832.2002.
29. **Köster W.** 2001. ABC transporter-mediated uptake of iron, siderophores, heme and vitamin B12. Res Microbiol **152:**291–301. http://dx.doi.org/10.1016/S0923-2508(01)01200-1.
30. **Höfte M.** 1992. Classes of microbial siderophores, p 3–26. *In* Bart L, Hemming B (ed), Iron chelation in plants and soil microorganisms. Academic Press, New York, NY.
31. **Jin B, Newton SMC, Shao Y, Jiang X, Charbit A, Klebba PE.** 2006. Iron acquisition systems for ferric hydroxamates, haemin and haemoglobin in Listeria monocytogenes. Mol Microbiol **59:**1185–1198. http://dx.doi.org/10.1111/j.1365-2958.2005.05015.x.
32. **Sebulsky MT, Hohnstein D, Hunter MD, Heinrichs DE.** 2000. Identification and characterization of a membrane permease involved in iron-hydroxamate transport in Staphylococcus aureus. J Bacteriol **182:**4394–4400. http://dx.doi.org/10.1128/JB.182.16.4394-4400.2000.
33. **Fecker L, Braun V.** 1983. Cloning and expression of the fhu genes involved in iron (III)-hydroxamate uptake by Escherichia coli. J Bacteriol **156:**1301–1314.
34. **Forman S, Nagiec MJ, Abney J, Perry RD, Fetherston JD.** 2007. Analysis of the aerobactin and ferric hydroxamate uptake systems of Yersinia pestis. Microbiology **153:**2332–2341. http://dx.doi.org/10.1099/mic.0.2006/004275-0.
35. **Zawadzka AM, Kim Y, Maltseva N, Nichiporuk R, Fan Y, Joachimiak A, Raymond KN.** 2009. Characterization of a Bacillus subtilis transporter for petrobactin, an anthrax stealth siderophore. Proc Natl Acad Sci U S A **106:**21854–21859. http://dx.doi.org/10.1073/pnas.0904793106.
36. **Hickford SJH, Küpper FC, Zhang G, Carrano CJ, Blunt JW, Butler A.** 2004. Petrobactin sulfonate, a new siderophore produced by the marine bacterium Marinobacter hydrocarbonoclasticus. J Nat Prod **67:**1897–1899. http://dx.doi.org/10.1021/np049823i.
37. **Homann VV, Edwards KJ, Webb EA, Butler A.** 2009. Siderophores of Marinobacter aquaeolei: petrobactin and its sulfonated derivatives. BioMetals **22:**565–571. http://dx.doi.org/10.1007/s10534-009-9237-0.
38. **He J, Miyazaki H, Anaya C, Yu F, Yeudall WA, Lewis JP.** 2006. Role of Porphyromonas gingivalis FeoB2 in metal uptake and oxidative stress protection. Infect Immun **74:**4214–4223. http://dx.doi.org/10.1128/IAI.00014-06.
39. **Fetherston JD, Mier I, Truszczynska H, Perry RD.** 2012. The Yfe and Feo transporters are involved in microaerobic growth and virulence of Yersinia pestis in bubonic plague. Infect Immun **80:**3880–3891. http://dx.doi.org/10.1128/IAI.00086-12.
40. **Weaver EA. Wyckoff EE, Mey AR, Morrison R, Payne SM.** 2013. FeoA and FeoC are essential components of the vibrio cholerae ferrous iron uptake system, and FeoC interacts with FeoB. J Bacteriol **195:**4826–4835. http://dx.doi.org/10.1128/JB.00738-13.
41. **Møller JV, Juul B, le Maire M.** 1996. Structural organization, ion transport, and energy transduction of P-type ATPases. Biochim Biophys Acta **1286:**1–51. http://dx.doi.org/10.1016/0304-4157(95)00017-8.
42. **Liuzzi JP, Cousins RJ.** 2004. Mammalian zinc transporters. Annu Rev

Nutr **24:**151–172. http://dx.doi.org/10.1146/annurev.nutr.24.012003.132402.
43. **Papp-Wallace KM, Maguire ME.** 2006. Manganese transport and the role of manganese in virulence. Annu Rev Microbiol **60:**187–209. http://dx.doi.org/10.1146/annurev.micro.60.080805.142149.
44. **Hantke K.** 2001. Iron and metal regulation in bacteria. Curr Opin Microbiol **4:**172–177. http://dx.doi.org/10.1016/S1369-5274(00)00184-3.
45. **Rensing C, Mitra B, Rosen BP.** 1997. The zntA gene of Escherichia coli encodes a Zn (II)-translocating P-type ATPase. Proc Natl Acad Sci U S A **94:**14326–14331. http://dx.doi.org/10.1073/pnas.94.26.14326.
46. **Grass G, Franke S, Taudte N, Nies DH, Kucharski LM, Maguire ME, Rensing C.** 2005. The metal permease ZupT from Escherichia coli is a transporter with a broad substrate spectrum. J Bacteriol **187:**1604–1611. http://dx.doi.org/10.1128/JB.187.5.1604-1611.2005.
47. **Moore CM, Helmann JD.** 2005. Metal ion homeostasis in Bacillus subtilis. Curr Opin Microbiol **8:**188–195. http://dx.doi.org/10.1016/j.mib.2005.02.007.
48. **Fuangthong M, Helmann JD.** 2003. Recognition of DNA by three ferric uptake regulator (Fur) homologs in Bacillus subtilis. J Bacteriol **185:**6348–6357. http://dx.doi.org/10.1128/JB.185.21.6348-6357.2003.
49. **Patzer SI, Hantke K.** 2001. Dual repression by Fe(2+)-Fur and Mn(2+)-MntR of the mntH gene, encoding an NRAMP-like Mn(2+) transporter in Escherichia coli. J Bacteriol **183:**4806–4813. http://dx.doi.org/10.1128/JB.183.16.4806-4813.2001.
50. **Anjem A, Varghese S, Imlay JA.** 2009. Manganese import is a key element of the OxyR response to hydrogen peroxide in Escherichia coli. Mol Microbiol **72:**844–858. http://dx.doi.org/10.1111/j.1365-2958.2009.06699.x.
51. **Yoch DC, Valentine RC.** 1972. Ferredoxins and flavodoxins of bacteria. Annu Rev Microbiol **26:**139–162. http://dx.doi.org/10.1146/annurev.mi.26.100172.001035.
52. **Witkin E.** 1976. Ultraviolet mutagenesis and inducible DNA repair in Escherichia coli. Bacteriol Rev **40:**869–907.
53. **Gilbreath JJ, West AL, Pich OQ, Carpenter BM, Michel S, Scott Merrell D.** 2012. Fur activates expression of the 2-oxoglutarate oxidoreductase genes (oorDABC) in Helicobacter pylori. J Bacteriol **194:**6490–6497. http://dx.doi.org/10.1128/JB.01226-12.
54. **Butcher J, Stintzi A.** 2013. The transcriptional landscape of Campylobacter jejuni under iron replete and iron limited growth conditions. PLoS One **8:**1–16.
55. **Janoir C, Denève C, Bouttier S, Barbut F, Hoys S, Caleechum L, Chapetón-Montes D, Pereira FC, Henriques AO, Collignon A, Monot M, Dupuy B.** 2013. Adaptive strategies and pathogenesis of Clostridium difficile from in vivo transcriptomics. Infect Immun **81:**3757–3769. http://dx.doi.org/10.1128/IAI.00515-13.