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Limited Role for Iron Regulation in *Coxiella burnetii* Pathogenesis▽†

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In gram-negative bacteria, iron acquisition proteins are commonly regulated by Fur (*f*erric *u*ptake *r*egulator), which binds iron-regulated promoters (the Fur box). We hypothesized that *Coxiella burnetii* requires iron and employs an iron-regulatory system and used various approaches to define a Fur regulon. Cloned *C. burnetii fur* complemented an *Escherichia coli fur* deletion mutant. A ferrous iron transporter gene (CBU1766), a putative iron binding protein-encoding gene (CBU0970), and a cation efflux pump gene (CBU1362) were identified by genome annotation and using a Fur titration assay. Bioinformatically predicted Fur box-containing promoters were tested for transcriptional control by iron. Five genes demonstrated at least a twofold induction with minimal iron. Putatively regulated genes were evaluated in a two-plasmid regulator/promoter heterologous expression system. These data suggested a very limited Fur-regulated system in *C. burnetii*. In an in vitro tissue culture model, a significant increase in bacterial growth was observed with infected cells treated with deferoxamine in comparison to growth under iron-replete conditions. In an iron-overloaded animal model in vivo, the level of bacterial growth detected in the iron-injected animals was significantly decreased in comparison to growth in control animals. In a low-iron-diet animal model, a significant increase in splenomegaly was observed, but no significant change in bacterial growth was identified. The small number of predicted iron acquisition systems, few Fur-regulated genes, and enhanced replication under a decreased iron level predict a requirement of a low level of iron for survival, perhaps to avoid creation of additional reactive oxygen radicals.

Coxiella burnetii, the etiologic agent of Q fever, is an obligate, intracellular, gram-negative bacterium. In vitro, it can replicate in a range of cell lines, including fibroblasts and macrophage-like cells (28). Transmission of bacteria occurs primarily by the aerosol route. Patients with acute Q fever typically present with fatigue, high fever, and periorbital headache, while chronic Q fever usually develops as endocarditis or hepatitis (42). The bacterium is found in a variety of animal reservoirs, such as arthropods, sheep, goats, and cattle (28). Therefore, Q fever is considered an occupational hazard for those in close contact with livestock (40). The Centers for Disease Control and Prevention have classified *C. burnetii* as a class B bioterrorism agent (13).

The ability to acquire iron is essential in the pathogenesis of the majority of infectious bacteria (4). Iron is used in functions as diverse as respiration, oxygen transport, and DNA biosynthesis (4). However, acquisition must be tightly regulated, since excess iron results in the generation of oxygen radicals via the Fenton and Haber-Weiss reactions. These oxygen radicals can cause damage in DNA and other critical macromolecular structures (60). Expression of iron acquisition proteins in bacteria is typically transcriptionally regulated by a repressor, described as the *f*erric *u*ptake *r*egulator (Fur) in *Escherichia coli* (26). Homologues in gram-positive and gram-negative bacteria, including phylogenetic relatives of *Coxiella*, the intracellular pathogenic *Legionella pneumophila*, have been described previously (4, 30). The prototypic model for transcriptional regulation is, in an iron-replete environment, Fur binding its corepressor, Fe^{2+}. The complex blocks transcription of Fur-regulated genes by binding to a palindromic sequence, which is known as a Fur box, present in the promoter region (19). This typically results in transcriptional repression. A few experimental models have noted activation of some promoters (17, 20). Masse et al. recently described a process of Fur activation via an indirect method involving a small RNA, the RyhB RNA (41).

C. burnetii replicates within a vacuole with properties similar to the phagolysosome of host cells, normally an inhospitable environment (8). It is currently unknown what nutrients are available in this milieu. Howe and Mallavia reported an upregulation of transferrin receptor expression during the first 24 h of infection, suggesting a correlation between iron acquisition and infection. The authors also predicted an absolute requirement for iron by describing inhibition of *C. burnetii* replication in J774A.1 murine macrophage-like cells incubated in the presence of deferoxamine, an iron chelator (33). We hypothesized that, similar to *Legionella* spp., *C. burnetii* employs a functional Fur regulon to maintain essential levels of iron.

In this paper, we describe the characterization of a *C. burnetii fur* homologue, which functionally complements an *E. coli fur* deletion strain. We identify a putative Fur-regulated gene (*frg1*) and a cation efflux pump (CBU1362) by using a Fur titration assay (FURTA) and an iron acquisition gene homologue, the ferrous iron transporter *feoB*, by analyzing the ge-

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nome annotation of *C. burnetii*. Previously, putative members of a Fur regulon were identified by bioinformatic screening of promoter regions for all *C. burnetii* open reading frames (ORFs). (*C. burnetii* ORFs identified by the CBU numbers presented in the text were assigned locus tags in The Institute for Genomic Research [TIGR] annotation [55].) Members of the putative Fur regulon were evaluated by transcriptional profiling of *C. burnetii* in axenic acid activation buffer and by a two-plasmid regulator/promoter heterologous system. We also predict a requirement of low levels of iron for *C. burnetii* replication in both tissue culture and animal models of infection. We conclude that *C. burnetii* likely encodes a limited set of iron transport systems to prevent oxidative damage associated with iron acquisition.

MATERIALS AND METHODS

Media and chemicals. Luria-Bertani (LB) medium (Difco, Detroit, MI) was prepared according to a laboratory manual (39). Antibiotics were incorporated into media at the following concentrations to maintain plasmids in *E. coli*: kanamycin, 50 μg ml^{-1}; tetracycline, 12.5 μg ml^{-1}; and chloramphenicol, 30 μg ml^{-1}. Purified *C. burnetii* was incubated at 37°C for two hours in an acid activation buffer. The buffer (32 mM KPO_4, 15 mM NaCl, 152.2 mM KCl, 100 mM glycine, 5 mM L-glutamate, 20 μM L-proline) was prepared at a pH 4.5 as previously described (32). $FeSO_4 \cdot 7H_2O$ was added to the buffer at the concentrations as indicated in Table 2. ONPG (*o*-nitrophenyl-β-D-galactopyranoside), deferoxamine, ferrous sulfate, and ferric chloride were purchased from Sigma Chemical Co. (St. Louis, MO). All *E. coli* cultures were grown in LB medium at 37°C on a shaker.

Propagation and isolation of *C. burnetii*. *C. burnetii* Nine Mile RSA 439 was maintained in a persistent infection of an L929 murine fibroblast cell line. Infected cells were incubated at 37°C with 5% CO_2. *C. burnetii* was freshly purified from infected cells as follows. Infected cells were pooled and lysed in double-distilled water. Cell debris was pelleted at 1,300 × *g*, for 5 min. The supernatant was centrifuged at 13,600 × *g* for 30 min, and the resulting pellet was resuspended in 0.25 M sucrose phosphate buffer (53.9 mM Na_2HPO_4, 12.8 mM KH_2PO_4, 72.6 mM NaCl, 0.25 M sucrose). The solution was centrifuged at 1,300 × *g* for 5 min to remove cellular debris. These two spins were alternated until minimal amounts of debris were detected. Bacteria were stored in 0.25 M sucrose phosphate buffer.

Isolation of DNA. Plasmid minipreps were prepared by using the FastPlasmid miniprep kit as described by the manufacturer (Eppendorf, Hamburg, Germany). The protocol for extraction of *C. burnetii* Nine Mile DNA from infected cells was as follows. Infected cells were resuspended in lysis buffer (0.25 M Tris [pH 7.5], 10 mM EDTA, 5 mM glucose, 200 mg lysozyme per 50 ml solution) with 1 mg/ml of proteinase K. Cells were incubated at 60°C for 4 h. Sodium dodecyl sulfate (SDS) was added at a final concentration of 1% and incubated at room temperature for 1 h. The protocol from High Pure PCR template preparation kit was performed as described by the manufacturer (Roche Molecular Biochemicals, Indianapolis, IN).

Plasmid construction. For the construction of pNP101 and pNP104, the *C. burnetii fur* sequence was cloned into pCR 2.1/TOPO (Invitrogen) by using the primers CbFur-For (CGGTTAGAGTGATGCCCARACAG) and CbFur-Rev (TGACCATAGGGTAAGAAGGCGTC) and designated pNP101. pNP101 was then digested with EcoRI (New England Biolabs, Ipswich, MA), resulting in an 820-bp fragment which was then cloned into pACYC184, and the construct was designated pNP104.

Primers for the two plasmid system (see Table S1 in the supplemental material) were used at a final concentration of 1 μM per 50-μl reaction volume. The amplification procedure consisted of 94°C for 1 min, 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 7 min, and 72°C for 6 min with a 4-s pause. Primers (Integrated DNA Technologies, Inc., Coralville, IA) (see Table S1 in the supplemental material) and *C. burnetii* RSA 493 (Nine Mile phase I) DNA were mixed with PCR SuperMix (Invitrogen, Carlsbad, CA), and reactions were carried out using the DNA Engine PT-200 Peltier thermal cycler (MJ Research, Reno, NV). PCR products were subsequently cloned into PCR cloning vector pBlue-TOPO (Invitrogen, Carlsbad, CA).

Real-time PCR. Standards were generated by 10-fold serial dilutions of 10^7 genome copies/μl. Two and a half μl of each standard were mixed with 12.5 μl SYBR green PCR master mix (Applied Biosystems, Foster City, CA), 1 μl of each primer, and 8 μl of distilled H_2O. Forward (GCACTATTTTTAGCCGG AACCTT) and reverse (TTGAGGAGAAAAACTGGATTGAGA) primers which amplify a 74-bp fragment of *com1*, a conserved *C. burnetii* gene (CBU1910), were used. Primer sequences for putative Fur-regulated genes are in Table S2 in the supplemental material (Integrated DNA Technologies, Inc., Coralville, IA). The amplification was performed in a sequence detector (PE Applied Biosystems, Foster City, CA) with the following program: 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 m, and 95°C for 15s with a 4-s pause. The data were analyzed using Sequence Detection System software, version 1.3 (Perkin Elmer, Branchburg, NJ).

DNA sequence analysis. DNA was sequenced at Gene Technologies Laboratories (Biology Department, Texas A&M University, College Station, TX). The genomic data for the survey and the sequence analysis was provided by TIGR. Sequence homologies were compared using MacVector (Oxford Molecular Company, Madison, WI) and BLAST programs.

Genomic library construction. *C. burnetii* genomic DNA library was constructed with HindIII digested chromosomal DNA fragments ligated with HindIII-digested λZapII as described in the λZapII cloning kit manual (Stratagene, La Jolla, CA). Bacteriophage λZapII was mixed with *E. coli* strain XL1-MRF′ and incubated on NZY agar plates to yield approximately 500 plaques per plate. Bacteriophage plaques were removed with sterile Pasteur pipettes, transferred to phage dilution buffer and plasmids were excised as described in the λZapII/EcoRI/CIAP cloning kit instruction manual (Stratagene, La Jolla, CA).

FURTA. The FURTA was performed with *E. coli* strain H1717, which contains a *lacZ* fusion reporter gene under the transcriptional control of the Fur-regulated *fhuF* gene promoter. This strain was transformed with plasmids constructed from a *C. burnetii* genomic library. The activity of this iron-regulated reporter gene was monitored by a standard protocol (57). Bacteria were grown on MacConkey agar petri plates either under iron deprivation conditions by the addition of 50 μm of dipyridyl, an iron chelator, or in an iron-replete environment with 50 μm $FeCl_3$ supplementation. Expression of the iron-regulated *lacZ* reporter gene caused formation of dark pink colonies which is contrasted with colorless colonies formed due to Fur-mediated repression of *fhuF*::*lacZ* under iron-replete conditions.

Regulator/promoter two-plasmid system. The Fur box sequence was cloned into a promoter trap vector, pBlue-TOPO, as described by the manufacturer (Invitrogen, Carlsbad, CA). The vector and the full *C. burnetii fur* clone were cotransformed into BN4020, an *E. coli fur* deletion strain (6). pBlue-TOPO was also cotransformed with pMH15, a plasmid containing the full *E. coli fur* (25). β-Galactosidase activity was assessed quantitatively for bacterial cultures as described previously (43). The substrate for LacZ hydrolysis in this assay was ONPG. $FeCl_3$ was added at a final concentration of 50 μM. Deferoxamine, an iron chelator, was added at a final concentration of 200 μM. Three independent culture samples were measured in triplicate. Statistical analysis was performed using the Student's *t* test.

RNA isolation. Freshly purified *C. burnetii* Nine Mile phase II was incubated at 37°C in the acid activation buffer for 2 h. Bacteria were pelleted at 13,600 × *g*, for 30 min and resuspended in 200 μl Tris-EDTA buffer, pH 8.0, with lysozyme (4 mg/ml) and 21 μl of 10% SDS. Bacteria were incubated at room temperature for 30 min. The RNeasy protocol was followed as described by the manufacturer (Qiagen, Valencia, CA). DNase treatment was performed as described by the product manufacturer (Ambion, Austin, TX) and confirmed by reverse transcriptase-negative reactions. Reverse transcription reactions with random hexamer primers were performed using the Taqman reverse transcription kit (Applied Biosystems, Foster City, CA). Three independent RNA samples for each concentration were taken and pooled for reverse transcriptase reaction (Applied Biosystems) and real-time PCR analysis (Applied Biosystems). The experiment was performed in triplicate. Changes in expression levels were calculated using cycle threshold (C_T) values from real-time PCR analysis and normalized to *com1* values (ABI protocol no. 2).

J774A.1, J774A.16, and J774D.9 murine macrophage experiments. J774A.1, J774A.16, and J774D.9 murine macrophage-like cell lines were used to assess replication rates of *C. burnetii* Nine Mile phase II in the absence and presence of deferoxamine, an iron chelator. Cells were plated at 10^5/ml in 24-well plates and then allowed to adhere for a minimum of 4 h. Macrophages were infected at 50:1 with tissue culture-purified *C. burnetii* phase II (RSA 439), incubated overnight at 37°C in 5% CO_2 and then washed three times with warm medium. Infected cells were then incubated with RPMI media containing 0 or 30 μM deferoxamine. Samples were taken at 0 h, 24 h, 48 h, and 72 h posttreatment. Quantification of *C. burnetii* genomes was determined by real-time PCR as previously described (10). Host cell viability remained above 80% as determined by Trypan blue exclusion.

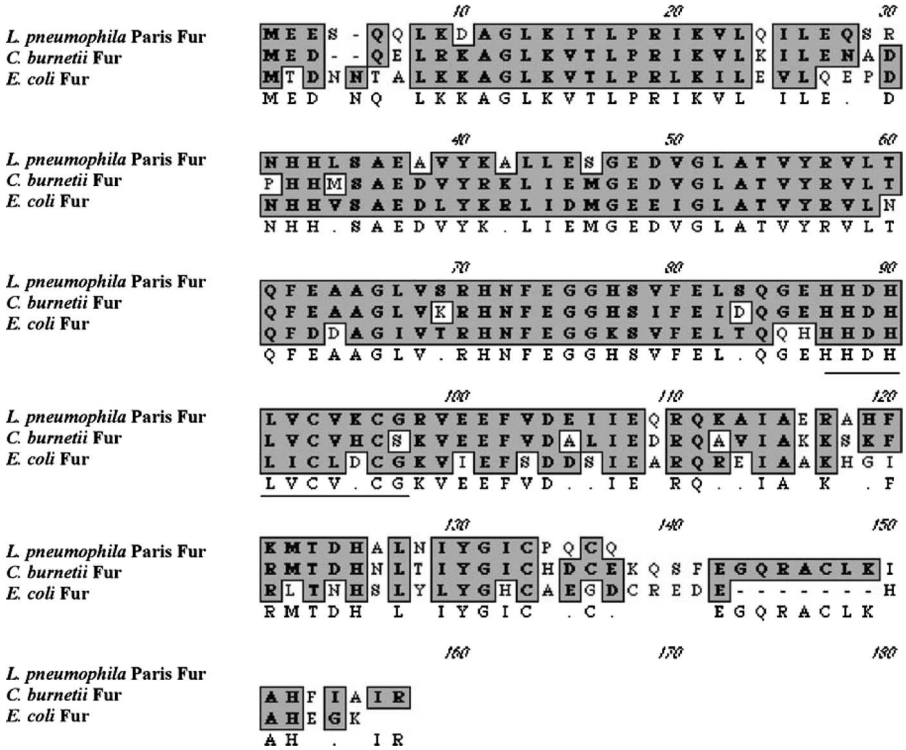

FIG. 1. ClustalW alignment of Fur homologues. Amino acid alignment of *C. burnetii* Fur with Fur homologues from *E. coli* K-12 and *L. pneumophila* Paris. The shaded areas are similarity matches among the species. The area underlined is the $HHXHX_2CX_2C$ motif (47).

***Legionella pneumophila*-infected J774A.1 cell experiment.** *L. pneumophila* was grown on buffered charcoal-yeast extract agar plates between 48 and 72 h. Tissue culture cells were plated at 5×10^5 cells/ml in 24-well tissue culture plates and incubated overnight at 37°C in 5% CO_2. Twenty-four hours after tissue culture seeding, bacteria were swabbed off the agar plate into buffered yeast extract broth. The optical density at 600 nm was measured, and J774A.1 cells were infected with *L. pneumophila* at a multiplicity of infection of 1:10. The infected cells were incubated for 1 h at 37°C in 5% CO_2. After incubation, the cells were washed with prewarmed phosphate-buffered saline, and fresh medium with 0 μM, 30 μM, or 50 μM deferoxamine was added. The cells were lysed in 1 ml of distilled water at 0 h, 24 h, and 48 h after deferoxamine treatment. The bacteria were centrifuged and the pellet was resuspended in buffered yeast extract broth. The samples were plated on buffered charcoal-yeast extract agar plates at 10× and 100× dilutions, and placed in a 37°C, 5% CO_2 incubator. CFU were visible and counted between 48 and 72 h.

***C. burnetii* infection in BALB/c mice.** Forty-eight female, six-week-old BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were divided into four groups of six mice each for two studies, utilizing iron deficiency and iron overload. Mice in group I served as negative controls with no experimental manipulation. Group II mice were fed an iron-deficient custom research diet (TD 80396; Harlan Teklad, Indianapolis, IN) ad libitum and were given deionized drinking water for two weeks prior to infection and throughout infection. Group III mice were also negative controls and received sterile saline injections intraperitoneally (i.p.) every other day for 2 weeks. Mice in group IV were iron overloaded by i.p. injection of 1.2 mg iron dextran (Vedco, St. Joseph, MO) every other day for two weeks prior to infection. Mice in groups I, III, and IV were provided a commercial rodent diet (Harlan Teklad, Indianapolis, IN) and water ad libitum.

All mice were infected i.p. with 10^7 phase I *C. burnetii* Nine Mile (RSA493) cells suspended in phosphate-buffered saline. Body weights were measured prechallenge and 14 days postchallenge. Animals in all groups were euthanized on day 14 postinfection by carbon dioxide overdose followed by exsanguination, and spleen weights were measured. Liver, spleen, and bone were collected at necropsy for histopathologic evaluation. Serum samples were collected for measurement of iron. All animal experimentation was reviewed and approved by Texas A&M University Laboratory Animal Care Committee and was performed in compliance with university and federal regulations. All animal experiments were conducted in a biosafety level III laboratory.

Quantification of *C. burnetii* in spleens. Approximately 0.025 g of spleen from each infected animal was homogenized in 500 μl lysis buffer (6.25 ml 2 M Tris, 1 ml 0.5 M EDTA, 50 mg glucose, 200 mg lysozyme). Homogenate (200 μl) was then incubated with 10 μl proteinase K for 4 h at 60°C. The lysate was then incubated with 21 μl 10% SDS at room temperature for 1 h. The High Pure PCR template preparation kit (Roche, Indianapolis, IN) was used to extract DNA, per the instructions of the manufacturer, following tissue lysis as previously described (2). *C. burnetii* genome copies were enumerated by real-time PCR using primers amplifying the *com1* gene as previously reported (10).

Measurement of serum iron. At day 14 postinfection, serum samples were collected. Serum iron levels were determined using the iron/total iron-binding capacity reagent set according to the manufacturer's protocol (Pointe Scientific, Canton, MI).

Calculation of bacterium generation time. $G = t/[3.3 \times \log(b/B)]$, where G is the generation time, t is the time interval (in hours), b is the genome copy number at the end of the interval, and B is the genome copy number at the start of the interval (http://textbookofbacteriology.net/growth.html).

Statistics. Significant differences between groups were determined by performing a two-tailed Student's t test. Significant P values were determined as described in Results.

RESULTS

Identification of *C. burnetii fur*. Since Howe and Mallavia suggested an absolute iron requirement for *C. burnetii* pathogenesis (33), we hypothesized that a Fur homologue may have a role in regulation of iron acquisition in *C. burnetii*. Analysis of the TIGR annotation of the *C. burnetii* Nine Mile genome revealed the nucleotide sequence for *fur* (CBU1301) (55). BLAST alignment of the annotated amino acid sequence of *C. burnetii* Fur showed 58% identity and 77% similarity to the *E. coli* K-12 Fur protein (accession number NP_415209) and 71% identity and 84% similarity to Fur from *L. pneumophila* (accession number CAH11586) (Fig. 1). Unlike the case for *fur*

TABLE 1. Tissue culture cells, bacterial strains, and plasmids used in this study

Cell line, strain, or plasmid	Description[a]	Source/reference
Cell lines		
L929	Murine fibroblast cell line	ATCC
J774A.1	Murine macrophage-like cell line	ATCC
J774A.16	Murine macrophage-like cell line with increased NADPH oxidase	14
J774D.9	Murine macrophage-like cell line without clonal NADPH oxidase	22
Strains		
Coxiella burnetii	Nine Mile, phase I, RSA 493, and Nine Mile, phase II, RSA 439	
Legionella pneumophila	AA100	53
Escherichia coli		
H1717	*aroD139 ΔlacU169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR aroB fhuF*::λ*placMu*	26
RRJC-1	*fur*::Tn*5fhuF*::*lacZ* in BN4020	59
DH5α	F$^-$ ϕ80d*lacZΔM15* Δ(*lacZYA-argF*)*U169 deoR recA endA1 phoA relA1*	Gibco/BRL
Top10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZΔM15 ΔlacX74 deoR recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
BL21(DE3)	F$^-$ *ompT hsdS* (r_B^- m_B^-) *gal, dcm, plysE*	Novagen
BN4020	*fur*::Tn*5*	59
XL1-MRF′		Stratagene
Plasmids		
pMH15	pACYC184 with *E. coli fur*	25
pCR 2.1/TOPO	TA cloning vector, Ap^r	Invitrogen
pBAD/TOPO	TA cloning vector, arabinose inducible	Invitrogen
pBlue TOPO	TA cloning vector, promoter trap	Invitrogen
p20R	λZapII clone containing CBU0970 (FURTA)	This work
p15R	λZapII clone containing CBU1362 (FURTA)	This work
pNP101	pCR2.1/TOPO with *C. burnetii fur*	This work
pNP104	pACYC184 with *C. burnetii fur*	This work
pFeoB Fur Box	pBlue TOPO with FeoB Fur box	This work

[a] Str^r, streptomycin resistant; Ap^r, ampicillin resistant.

homologues in other organisms, sequence analysis of the predicted promoter region did not predict a Fur box; therefore, autoregulation by Fur was not expected.

***C. burnetii* Fur complementation of an *E. coli fur* mutant.** Since the ability to generate a *C. burnetii* mutant is currently unavailable, the functionality of *C. burnetii* Fur was demonstrated by complementing an *E. coli fur* deletion strain containing an *fhuF*::*lacZ* reporter fusion (RRJC-1) (Table 1). *fhuF* is a Fur-regulated gene in *E. coli*, thus, heterologous Fur proteins can be evaluated. Promoter activity was assessed quantitatively (43).

The complemented strain containing *C. burnetii fur*- (pNP101) in the presence of iron showed a significant decrease in β-galactosidase activity compared to the β-galactosidase activity of uncomplemented *fur* mutant (RRJC-1) ($P = 0.001$) (Fig. 2). To confirm iron as the regulatory cofactor in the complemented strain, 200 μM deferoxamine, an iron chelator, was added to the cultures. Under these conditions, derepression of *fhuF* was observed by a significant increase in β-galactosidase activity compared to that in the iron-containing samples ($P < 0.01$) (Fig. 2). As a positive control, the *E. coli fur* gene, carried on pMH15 (Table 1), was also transformed into RRJC-1, and a decrease in β-galactosidase activity in the presence of iron ($P < 0.01$) (Fig. 2) was observed.

Identification of Fur-regulated genes in the FURTA. Since *C. burnetii fur* complemented an *E. coli fur* deletion mutant, we then identified putative *C. burnetii* Fur-regulated genes. Initially, we used the FURTA with the *E. coli* strain (H1717). The FURTA uses the binding of the Fur-iron complex to the palindromic sequence (Fur box) in the promoter region to identify Fur-regulated genes in *E. coli* (57). Since *E. coli* Fur has shown the ability to bind to Fur boxes in other organisms, the FURTA has been successfully used to identify Fur-regulated genes of several bacteria (7, 21, 27). The FURTA reporter *E. coli* strain H1717 contains a chromosomal copy of *E. coli fur* and a *fhuF*::*lacZ* reporter fusion. Two independently generated *C.*

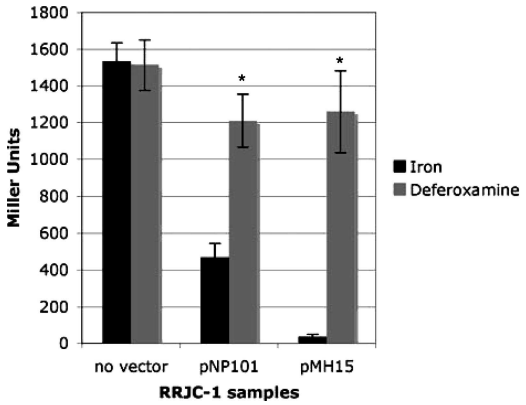

FIG. 2. *C. burnetii fur* complements an *E. coli fur* deletion mutant. *C. burnetii fur*(pNP101) was transformed into an *E. coli fur* mutant. Cultures were grown in the presence of iron or the iron chelator deferoxamine (200 μM). The *fhuF*::*lacZ* reporter fusion activity is reported as Miller units. *C. burnetii* Fur complemented the mutant and showed a significant decrease in activity in the presence of iron (the asterisk indicates a *P* value of <0.01). Deregulation of *C. burnetii* Fur was observed in the iron-depleted samples (the asterisk indicates a *P* value of <0.01).

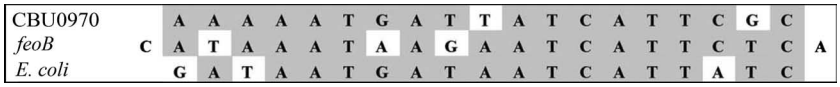

FIG. 3. ClustalW alignment of putative Fur boxes of *C. burnetii* genes. Amino acid sequence alignment of the Fur boxes of *frg1* (CBU0970), *feoB* (CBU1766), and the *E. coli* Fur box consensus. *frg1* (CBU0970) is 74% identical to the *E. coli* Fur box consensus, and *feoB* (CBU1766) is 68% identical to this consensus.

burnetii restriction fragment libraries were transformed into H1717. Plasmid inserts containing putative Fur boxes were identified by a derepression of the *fhuF*::*lacZ* fusion in the presence of iron (50 μM $FeCl_2$), as Fur preferentially bound to the numerous copies of the insert.

Unexpectedly, only two genes were identified in this assay (CBU0970 and CBU1362), although over 20,000 clones were screened from two independently generated libraries. We designated CBU0970 *frg1* (*f*ur-*r*egulated *g*ene *1*). With the TIGR Nine Mile annotated sequence, a BLAST analysis did not reveal any significant homology for *frg1* to previously characterized genes in the database. Using SignalP-HMM amino acid sequence analysis, a signal sequence was predicted between M1 and A26 (45, 46). Amino acid sequence analysis also identified a histidine-rich region at H52 to H60 and two possible metal binding sites, C118 to C123 (CX_4C) and C260 to C264 (CX_3C). Sequence analysis also revealed a putative Fur box in the promoter region with 74% identity to the *E. coli* Fur box consensus sequence (Fig. 3) (57).

BLAST analysis of the CBU1362 annotated sequence predicted similarity to a family of cation efflux proteins. Members of this family belong to the larger group of cation diffusion facilitator (CDF) proteins and are primarily involved in metal ion transport (5). ClustalW sequence analysis of the promoter region predicted a region with 58% identity to the *E. coli* Fur box consensus sequence (57).

Survey of the *C. burnetii* Nine Mile annotated genome. We recently reported the identification of *feoB* (CBU1766), a ferrous iron transporter gene, in the genome annotation and the initial characterization of *C. burnetii* Fur regulation of the gene (11). FeoB has been previously described as a Fur-regulated ferrous iron acquisition protein in *E. coli* (34). A BLASTP alignment of the predicted amino acid sequence of *C. burnetii* FeoB indicated that it was 47% identical and 66% similar to the *L. pneumophila* Paris FeoB protein (data not shown). ClustalW alignment of *feoB* Fur Box and *E. coli* Fur box consensus sequences showed that the sequences were 68% identical and that the *feoB* Fur box was 74% identical to *frg1* putative Fur box (Fig. 3).

In addition, a ferripyochelin binding protein (encoded by CBUA0035) was annotated on the QpH1 plasmid. However, this ORF contains a frameshift mutation, which predicts a nonfunctional protein. One other putative siderophore gene was identified, a homologue to *L. pneumophila frgA*, a gene with homology to aerobactin synthetase (29). However, unlike *L. pneumophila*, the *C. burnetii frgA* did not have a predicted Fur box, and the operon associated with nonclassical siderophore production in *L. pneumophila*, *lbtAB*, encoding a siderophore synthetase and a predicted transporter, was not annotated or found in the *C. burnetii* genome (1). Therefore, *frgA* is not a likely Fur-regulated candidate. Unexpectedly, a TonB homologue, associated with classical siderophore production, was not annotated, supporting the hypothesis that *C. burnetii* does not have the ability to import ferric iron through typical siderophores. Data from whole-genome sequence annotation suggest that transport of ferrous iron by *feoB* serves as the primary method of iron acquisition by *C. burnetii*.

We previously identified 18 putative Fur-regulated genes by an in silico approach (11). Briefly, the *C. burnetii* genome was searched using the *E. coli* Fur box consensus sequence and the putative Fur boxes of *feoB* and *frg1*. Nucleotides in the putative promoter regions matched at least 50% of the nucleotides in one or more of the three Fur boxes. These regions were also within at least 100 bases of the predicted start site.

Transcriptional analysis of putative Fur-regulated genes. We screened the putative Fur-regulated genes by using an axenic acid activation buffer system. Previously, this method has been used to analyze metabolically active, nonreplicating *C. burnetii* for protein expression (32, 33). In this system, the iron levels can be tightly controlled in comparison to the case when using infected cells incubated with deferoxamine. Another advantage is the ability to analyze bacterial response to iron without the presence of host factors.

To test iron regulation, gene transcript levels were measured under iron-replete and iron-depleted conditions. First, *C. burnetii* Nine Mile phase II was freshly purified from persistently infected L929 murine fibroblast cells. The bacteria were incubated in acid activation buffer at pH 4.5 (32) for two hours in buffer alone (trace iron) or with 10 μM or 50 μM ferrous sulfate added. The transcript level of each gene with specific primers was compared with C_T value of the control, *com* (subtracted from average C_T value of each test gene to normalize) (data not shown). To demonstrate expression level change, the 10 μM samples were used for the baseline. Calculations were based on the difference between baseline and trace iron (no additional iron) or high iron levels (50 μM).

As positive controls, Fur protein levels were evaluated in the acid activation system to ensure the regulator was present. Freshly purified *C. burnetii* Nine Mile phase II was incubated in the acid activation buffer at pH 4.5 with trace levels of ferrous sulfate and 50 μM ferrous sulfate. The Fur protein was detected in the bacterial lysates by using a polyclonal rabbit CbFur antibody. As a control for equal loading, Com1, a constitutively expressed protein in *C. burnetii*, was detected using a mouse monoclonal antibody (available in our laboratory) (see Fig. S1 in the supplemental material).

As a negative control for the experimental design, we evaluated the transcriptional regulation of *fur* (CBU1301) by iron. As previously described, a Fur box was not predicted in the promoter region of the annotated sequence, and therefore, it was predicted not to be regulated by iron. No significant change was observed under the high-iron-concentration condition compared to the observations under the low-iron-concentration condition (Table 2).

TABLE 2. Expression of putative Fur-regulated genes

Locus tag (gene)	Group[a]	Fold change in expression for gene with:	
		50 μM $FeSO_4$	No iron added
CBU0216	I	0.77 ± 0.32	2.6 ± 0.7
CBU1106	I	1.0 ± 0.4	3.9 ± 4
CBU0769	II	1.2 ± 0.1	1.2 ± 0.5
CBU1477	II	4.5 ± 5	1.3 ± 0.9
CBU1546	II	1.0 ± 0.4	1.0 ± 0.3
CBU0280	II	1.3 ± 0.4	1.3 ± 0.5
CBU0395	III	1.0 ± 0.3	1.3 ± 0.2
CBU0432	III	1.2 ± 0.2	1.3 ± 0.3
CBU0611	III	1.0 ± 0.1	1.0 ± 0.2
CBU0920	III	1.0 ± 0.1	1.2 ± 0.3
CBU0968	III	1.0 ± 0.3	2.7 ± 0.8
CBU0970 (*frg1*)	III	1.0 ± 0.1	2.8 ± 0.7
CBU1362	III	1.1 ± 0.3	1.0 ± 0.2
CBU1493	III	1.0 ± 0.3	1.5 ± 0.3
CBU1556	III	1.0 ± 0.1	0.77 ± 0.1
CBU1600	III	1.1 ± 0.3	0.93 ± 0.2
CBU1695	III	1.0 ± 0.3	0.81 ± 0.1
CBU1851	III	0.9 ± 0.1	1.3 ± 0.3
CBU2012	III	1.1 ± 0.1	1.0 ± 0.2
CBU0963	IV	1.2 ± 0.2	1.2 ± 0.3
CBU1766 (*feoB*)	IV	0.78 ± 0.1	3.7 ± 1.1
CBU1301 (*fur*)	IV	1.3 ± .1	1.3 ± 0.5

[a] Genome copy numbers were as follows: for group I, 10 to 1×10^2; for group II, 1×10^2 to 1×10^3; for group III, 1×10^3 to 1×10^4; for group IV, 1×10^4 to 1×10^5.

We predicted that *feoB* (CBU1766), as it has been previously identified in both *L. pneumophila* and *E. coli* as being Fur regulated, would be regulated by iron in *C. burnetii* (34, 52). As expected, a 3.7-fold induction of the transcript level for *feoB* in buffer alone was observed in comparison with the level in buffer supplemented with 50 μM ferrous sulfate (Table 2). This suggested that in the presence of a very low iron concentration (i.e., the trace level), the low level of corepressor allowed the induction of the gene. This concept of iron as a repressor was supported by the repression of the transcript in the presence of iron. By use of these changes as a comparison, the remaining putative Fur regulated genes were evaluated by transcriptional analysis.

The genes predicted by the FURTA were CBU0970 (*frg1*) and CBU1362. Similar to the case for *feoB*, a significant 2.8-fold induction of *frg1* was observed with buffer alone, and no change was observed with iron (Table 2). This further supported the FURTA prediction of Fur regulation. However, unlike *frg1*, CBU1362 transcript levels showed no significant change in either buffer alone or the presence of iron (Table 2). This suggested that CBU1362, a predicted cation efflux pump gene, is not regulated by iron and, therefore, not a likely Fur-regulated candidate. A positive result in the FURTA screen can also occur if overexpression of the protein results in a decrease in intracellular iron levels (57), and this may occur with CBU1362, as it is a predicted efflux pump.

Few of the other Fur-regulated candidates (CBU0216, CBU1106, and CBU0968) showed a significant induction of twofold or higher in buffer alone (Table 2). Interestingly, the intergenic region between CBU0968 and CBU0970 (*frg1*) is small, and a palindromic Fur box may be shared between the two genes. Unexpectedly, CBU1477 had a 4.5-fold induction with 50 μM ferrous sulfate. However, the standard deviation was large compared to the standard deviations for other members of the group, suggesting a transcriptional response other than iron regulation.

Relative expression levels of genes were also compared based on C_T values. C_T values were converted to transcript copy numbers as previously described (10). Group I indicates the lowest expression level (10 to 100 copies of transcript), and group IV represents the highest (10,000 to 100,000 copies of transcript). CBU0216 and CBU1106 are in group I, CBU0968 and CBU0970 are in group III, and *feoB* is located within group IV.

Analysis of *C. burnetii* Fur-Fur box interaction. While *feoB* (CBU1766), *frg1* (CBU0970), CBU0216, CBU1106, and CBU0968 were predicted as being transcriptionally regulated by iron, the acid activation buffer system did not evaluate Fur regulation. To indirectly assess *C. burnetii* Fur interaction with the putative Fur boxes, a heterologous expression system was developed by using a two-plasmid regulator/promoter system in an *E. coli fur* mutant (11). This method also allowed for an indirect comparison of regulatory activities of *C. burnetii* Fur and *E. coli* Fur. The pBlue-TOPO vector containing the putative Fur box region was cotransformed with a plasmid containing *C. burnetii* Fur (pNP104) into BN4020, an *E. coli fur* deletion strain. Deferoxamine, an iron chelator, was used to deplete the level of iron in the growing cultures. The plasmid pMH15 containing the full *E. coli fur* gene was used for a comparison, since it has been shown that *E. coli* Fur can interact with Fur boxes in other bacteria (18, 57). We confirmed the production of *C. burnetii* Fur in the system by Western blotting using polyclonal rabbit *C. burnetii* Fur antibody (see Fig. S2 in the supplemental material).

The insert in the pBlue-TOPO vector varied in size from clone to clone. The size was dependent on the primers designed, based on the annotated sequence. Several of the promoter regions proved difficult to clone, which could have been due to AT-rich regions in the upstream sequence. The clones were tested in three independent experiments performed in triplicate. We chose two levels of regulation by *t* test statistical analysis: weak regulation, predicted by a P value of ≤ 0.01, and strong regulation, predicted by a P value of ≤ 0.005 (Table 3).

The region containing the *feoB* Fur box was used as a positive control in the development of the assay (11). As predicted, it was strongly regulated by *C. burnetii* Fur (Table 3). It was also strongly regulated by *E. coli* Fur. This supported our approach of multiple identification methods to elucidate regulatory pathways.

Four other genes were predicted to be regulated by iron in the transcriptional screen: *frg1* (CBU0970), CBU0216, CBU1106, and CBU0968. *frg1* (CBU0970) was weakly regulated by *C. burnetii* Fur. In support of the FURTA data, it was strongly regulated by *E. coli* Fur (Table 3). However, the growth of the transformant containing *C. burnetii* Fur (pNP104 transformed into BN4020 with a pCBU970 Fur box) was inconsistent. The degree of promoter activity, as determined in Miller units, was close to background levels, which complicated the interpretation of this gene with regard to its Fur regulation. A similar issue occurred with the CBU0968 transformant containing *C. burnetii* Fur. However, it was also only weakly reg-

TABLE 3. Two-plasmid regulator/promoter analysis[a]

Locus tag (gene) or condition	Iron regulation[b]	*C. burnetii* Fur			*E. coli* Fur		
		Promoter activity (Miller units)		% Repression by iron	Promoter activity (Miller units)		% Repression by iron
		With iron	Without iron		With iron	Without iron	
Vector alone	NA	71 ± 10	71 ± 1	0	43 ± 3	58 ± 6	20
CBU0216	Yes	952 ± 121	1,163 ± 216	18	326 ± 29	397 ± 48	18
CBU1106	Yes	185 ± 11	183 ± 28	−1	88 ± 10	120 ± 11	27
CBU0769	No	512 ± 38	715 ± 65*	28	215 ± 53	593 ± 37**	64
CBU0280	No	689 ± 47	551 ± 62	−27	1,806 ± 193	1,846 ± 44	2
CBU0395	No	ND	ND		391 ± 65	394 ± 58	1
CBU0432	No	530 ± 22	845 ± 35*	37	803 ± 25	1,232 ± 23**	35
CBU0968	Yes	ND	ND		464 ± 43	843 ± 22*	45
CBU0970 (*frg1*)	Yes	78 ± 4	92 ± 2	15	415 ± 13	705 ± 32**	41
CBU1493	No	210 ± 8	256 ± 18	18	219 ± 21	249 ± 17	18
CBU1556	No	ND	ND		268 ± 9	345 ± 15*	22
CBU1695	No	ND	ND		1,112 ± 85	1,479 ± 121	25
CBU2012	No	253 ± 36	249 ± 15	−2	215 ± 35	251 ± 36	14
CBU1766 (*feoB*)	Yes	974 ± 45	3,212 ± 103**	70	193 ± 30	992 ± 42**	81

[a] Genes with significantly upregulated expression levels are indicated in boldface. *, *P* value in comparison to the value corresponding to the presence of iron, ≤0.01; **, *P* value in comparison to the value corresponding to the presence of iron, ≤0.005; ND, not determined.
[b] Predictions based on transcriptional screen.

ulated by *E. coli*, suggesting that of the two closely situated genes, *frg1* was the likely Fur-regulated candidate.

Neither CBU0216 nor CBU1106 was strongly regulated by either *C. burnetii* Fur or *E. coli* Fur (Table 3). This suggests that these genes may belong to an iron-regulatory pathway independent of Fur regulation. Also, as a control for the assay, some of the genes predicted not to be regulated by iron were screened in the two-plasmid system. Surprisingly, CBU0432 and CBU0769 were both weakly repressed by *C. burnetii* Fur and strongly regulated by *E. coli*. The CBU1556 transformant containing *C. burnetii* Fur (pNP104 transformed into BN4020 with a pCBU1556 Fur box) exhibited levels similar to those observed for CBU0970. It was weakly regulated by *E. coli* Fur and was not evaluated for *C. burnetii* Fur regulation. CBU0280, CBU1493, and CBU2012 were not predicted to be iron regulated, and Fur regulation was not demonstrated (Table 3).

Replication of *C. burnetii* in the presence of deferoxamine. With one annotated iron acquisition system in the genome and few additional Fur-regulated genes identified in our screens, we hypothesized that iron may play a less prominent role in *C. burnetii* replication than previously suggested by Howe and Mallavia (33). To test this hypothesis, we assessed the role of iron in three cell lines, i.e., J774A.1, J774D.9, and J774A.16, by treatment with deferoxamine, an iron chelator.

As a control for the experimental design, Trypan blue exclusion was used to assess viability of the cells in the presence of deferoxamine. Live cells were counted at 0 h, 24 h, 48 h, and 72 h posttreatment (see Fig. S3A in the supplemental material). While cell viability remained above 80% at all concentrations, it was noted that replication appeared to slow between 48 to 72 h posttreatment with 100 μM deferoxamine. This observation supports the data reported by Howe and Mallavia (33). Based on these results, we used 50 μM deferoxamine as an upper limit for treatment.

As another control for the experimental design, J774A.1 cells were infected with *L. pneumophila*, since it had been previously demonstrated that *L. pneumophila* is dependent on iron for growth (12). J774A.1 murine macrophage-like cells were infected with *L. pneumophila* and incubated with 0 μM, 30 μM, or 50 μM deferoxamine. Samples were taken at time zero and 24 h and 48 h after deferoxamine treatment. Compared to its replication rate in the absence of treatment, the replication rates of *L. pneumophila* over 24-h and 48-h time periods after deferoxamine treatment were significantly reduced (P value ≤ 0.05) (see Fig. S4 in the supplemental material).

J774A.1 cells were infected with *C. burnetii* Nine Mile phase II RSA 493 (avirulent passage variant of RSA 439) and allowed to infect overnight. The cells were then washed to remove uninternalized bacteria and incubated in medium containing deferoxamine. Samples were taken at time zero and 24 h, 48 h, and 72 h posttreatment. A significant increase (P value ≤ 0.01) in the genome copy number within the host cell in the presence of 30 μM deferoxamine was observed at 48 h and 72 h posttreatment compared to the number for the control (Fig. 4A). A significant decrease in generation time was observed for cells in the presence of 30 μM deferoxamine between 24 h and 72 h posttreatment (G = 10 h) and the untreated controls (G = 19 h) (P value = 0.01) (Fig. 4A). Interestingly, in all three experiments, the samples treated with 50 μM deferoxamine were inconsistent in the genome copy number, suggesting a possible modest effect to the host. Therefore, in the following experiments, we used 30 μM deferoxamine as the upper limit of treatment.

Based on these results, we theorized that by decreasing the availability of iron, the level of oxidative stress was decreased in response to the corresponding decrease in the Fenton reaction products. We therefore hypothesized that in an environment with a low level of oxidative stress, a decreased availability of iron should have minimal effect on *C. burnetii* replication. To test this hypothesis, we evaluated bacterial replication in the presence of deferoxamine in cell lines with different levels of oxidative stress as determined by the amount of expressed NADPH oxidase. J774A.16 is a murine macrophage-like cell line with an increased expression of NADPH oxidase, and J774D.9 is a cell line that is unable to express NADPH oxidase

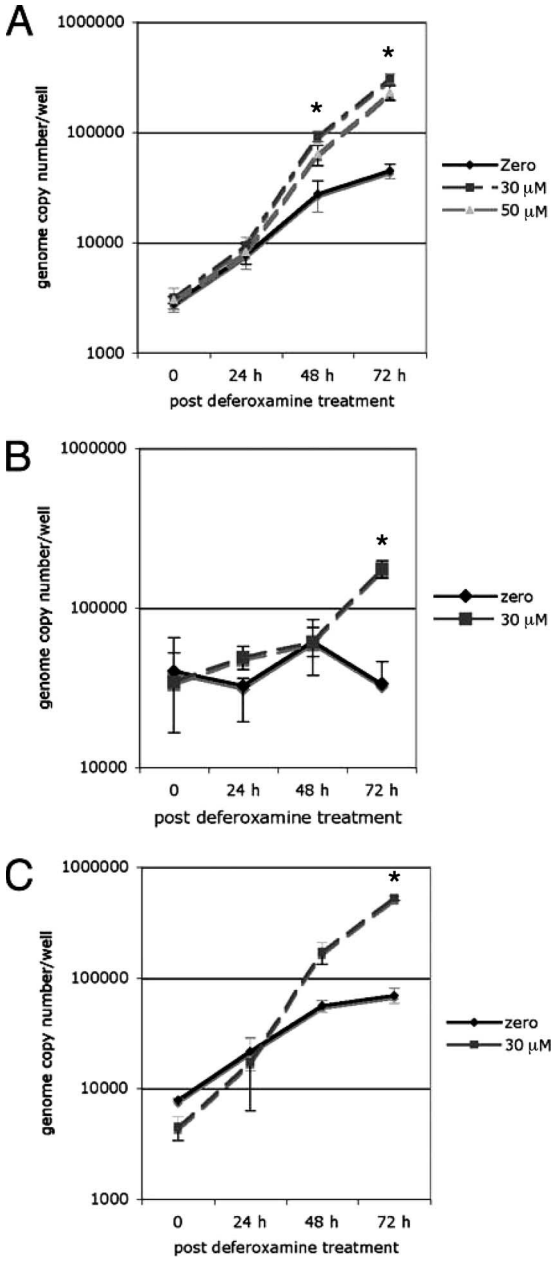

FIG. 4. *C. burnetii*-infected cells treated with deferoxamine. Cell lines were infected with *C. burnetii* phase II at a multiplicity of infection of 50:1. After being allowed to adhere overnight, cells were washed and treated with deferoxamine. Samples were taken at time zero, 24 h, 48 h, and 72 h. The data are representative of the trend observed in three independent experiments performed in triplicate. *C. burnetii* growth was assessed by genome copy number. (A) J774A.1 cells (the asterisk indicates a P value of ≤ 0.01). (B) J774A.16 cells (the asterisk indicates a P value of ≤ 0.01). (C) J774D.9 cells (the asterisk indicates a P value of ≤ 0.001).

(14, 22). Trypan blue exclusion was used to evaluate viability of J774A.16 and J774D.9 cells in the presence of deferoxamine. Viability remained above 85% for both cell lines under all conditions (see Fig. S3B and C in the supplemental material).

J774A.16 and J774D.9 cells were infected with *C. burnetii* Nine Mile phase II as in the J774A.1 experiment. As predicted, at 48 h in the J774A.16 control samples (with increased NADPH oxidase), the genome copy started to decrease, which indicated poor growth (Fig. 4B). At 48 h, the genome copy numbers in deferoxamine-treated J774A.16 samples continued to increase significantly (P value ≤ 0.01) (Fig. 4B). In J774D.9 samples (no clonal NADPH oxidase), a trend similar to that observed in J774A.1 cells was apparent (P value ≤ 0.001) (Fig. 4C).

Replication of *C. burnetii* in iron-overloaded and decreased-iron animal models. The increased replication of *C. burnetii* in the tissue culture infection model was intriguing and atypical of bacterial pathogenesis. To test whether this phenotype was consistent in vivo, iron-overloaded and decreased-iron animal models were used to evaluate *C. burnetii* infection in these environments with different iron levels. In the iron-overloaded models of infection, an increased bacterial load has been demonstrated in other bacteria, such as *Salmonella enterica* serovar Typhimurium (54), *Mycobacterium tuberculosis* (38), and *Vibrio vulnificus* (63). We hypothesized that, unlike most bacteria, in the decreased-iron model, an enhanced infection would be observed, and no significant changes in infection would be observed in the iron overload model. We injected BALB/c mice with either iron dextran or saline three times a week for two weeks prior to infection. Iron dextran has been shown to be nontoxic to mice, unlike iron sulfate, ferric ammonium citrate, and iron sorbital citrate (31). After infection with *C. burnetii* Nine Mile RSA 439, the mice did not receive any further injections of iron. *C. burnetii* infection was evaluated by splenomegaly (64) and quantification of bacterial growth by enumerating genome copy number (3, 10).

BALB/c mice were infected with a high dose (10^7) of *C. burnetii* Nine Mile phase I, and samples were taken on day 14 postinfection. For a control, we measured the serum iron levels of the mice on day 14 postinfection. The iron-injected group had a 41% increase (P value = 0.0008) in serum iron levels compared to the levels for the normal saline-injected group of infected mice. A similar increase was observed for control mice. We also evaluated the iron loads in the spleen and liver visually by using Prussian blue stain, which stains ferric iron (56). There was a readily observable increase in the amounts of staining between the saline- and iron-injected mice in both livers and spleens (see Fig. S5 and S6 in the supplemental material). Of note, it appeared that the iron was concentrated in granuloma formations in the livers of infected mice, possibly due to the increased prevalence of macrophages (see Fig. S6 in the supplemental material). The iron-injected group of infected mice had no significant increase in splenomegaly (P value = 0.73) compared to saline-injected group of infected mice (Fig. 5A). However, there was a significant decrease in the genome copy number (P value = 0.02) of the iron-overloaded group compared to the value for the control (Fig. 5B).

We then evaluated *C. burnetii* infection with lower nutritionally regulated levels of iron. In a previous study in 1999, Gomes et al. compared the efficacies associated with using an iron chelator, deferoxamine, and with a low-iron diet to develop the iron-deficient mouse model of infection (23). In a C57BL/6 mouse on a low-iron diet, serum iron levels were decreased compared to control levels, and replication of *M. avium* also decreased. In the deferoxamine-treated C57BL/6 animals, the serum iron levels were not lower than control levels and had no

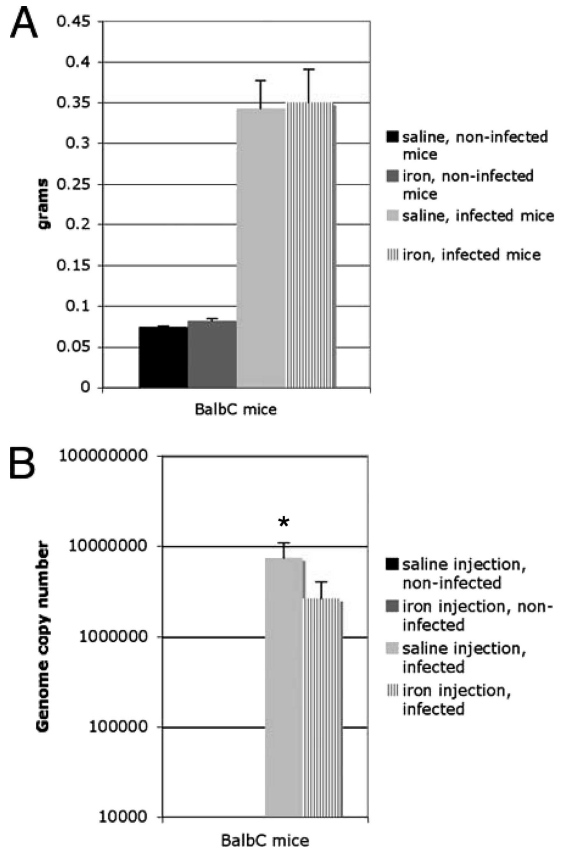

FIG. 5. Iron-overloaded animal model. Mice (five per group) were injected with either iron dextran or saline for two weeks prior to infection with *C. burnetii* Nine Mile phase I. Samples were taken at day 14 postinfection. (A) Measure of splenomegaly. (B) Quantification of *C. burnetii* genome copy number in spleens (the asterisk indicates a *P* value of 0.02).

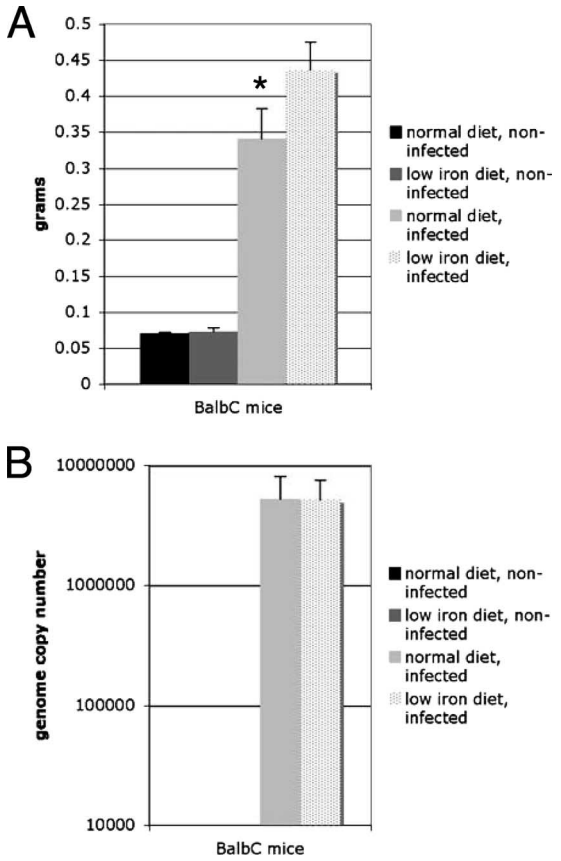

FIG. 6. Decreased-iron animal model. Mice (five per group) were fed either a decreased-iron diet or a normal diet for two weeks prior to and throughout infection with *C. burnetii* Nine Mile phase I. (A) Measure of splenomegaly (the asterisk indicates a *P* value of 0.0007). (B) Quantification of *C. burnetii* genome copy number in spleens.

effect on the bacterial growth rate, indicating that for a long-term experiment, a low-iron diet would be more effective in lowering iron concentrations (23).

As in the iron overload model, we infected BALB/c mice with a high dose (10^7) of *C. burnetii* Nine Mile phase I. Mice were fed a low-iron diet and deionized water for two weeks prior to infection and throughout the duration of the infection. As a control, the serum iron levels were measured at day 14 postinfection. The low-iron, infected group had a 39% decrease (*P* value = 0.005) in serum iron levels compared to those of the infected group on a normal diet. The control mice had serum iron levels similar those of the low-iron, infected group. With the Prussian blue stain, there were low observable levels of iron detected in both the spleen and the liver of the mice with decreased-iron diet (see Fig. S7 and S8 in the supplemental material). Granuloma formation was observed in the livers of infected mice, but the increase in iron was not as apparent as in the iron-overloaded animal model. On day 14 postinfection, the infected mice on the low-iron diet had a significant increase in splenomegaly (*P* value = 0.0007) compared to that of the controls (Fig. 6A). However, there was no significant difference (*P* value = 0.87) between the genome copy numbers of the mice on the low-iron diet and those on the normal diet (Fig. 6B).

DISCUSSION

In this study, we describe the identification and initial characterization of *C. burnetii fur*, the genes comprising the *C. burnetii* Fur regulon, and a limited role for iron during *C. burnetii* pathogenesis. The TIGR annotation of *C. burnetii* Nine Mile phase I predicted a *fur* homologue. Based on sequence analysis of the entire *fur* gene, a close similarity between *L. pneumophila fur* and *C. burnetii fur* was demonstrated. A challenge in analyzing gene regulation in *C. burnetii* is the inability to isolate isogenic mutants. For this reason, we chose to use an *E. coli fur* deletion strain (RRJC-1) to evaluate the functionality of *C. burnetii* Fur. Within this heterologous system, *C. burnetii* Fur, in the presence of iron, repressed the *E. coli* Fur-regulated *fhuF*::*lacZ* fusion. This repression was reversed with iron chelation, indicating that the *C. burnetii* Fur can function as a Fur-like regulatory protein.

A regulatory difference was observed in the interaction between *C. burnetii fur* and the *E. coli* Fur-regulated *fhuF*::*lacZ* fusion compared to *E. coli fur* (Fig. 2). This was supported by observations of *E. coli fur* and *C. burnetii fur* in the indirect

evaluation of Fur-Fur box interaction in the two plasmid regulator/promoter heterologous system (Table 3). ClustalW analysis of the amino acid sequences from *C. burnetii* and *E. coli* did not reveal any significant amino acid differences in the predicted N-terminal DNA-binding domain but did identify differences in the C-terminal dimerization domain which may affect function (Fig. 1). Within the predicted metal binding motif $HHXHX_2CX_2C$ (H87 to C95), the glycine residue at the end of the motif in *E. coli* is replaced with a serine residue in *C. burnetii* (Fig. 1) (47). It is interesting that the glycine is also conserved in *L. pneumophila fur*. This motif is also predicted to be the iron-regulatory binding site, and this change in residues may be responsible for the differences in regulation observed with *C. burnetii* Fur (50).

Demonstration of a functional *C. burnetii* Fur suggested that a Fur regulon was present in the obligate intracellular pathogen. In some gram-negative bacteria, the Fur protein is autoregulated, and a Fur box has been identified in the promoter region of the *fur* gene (15, 16). In addition to autoregulation, Fur homologues have been described as being regulated, either directly or indirectly, by transcriptional regulators such as OxyR and RpoS (36, 65). Sequence analysis of *C. burnetii fur* did not predict a Fur box in its upstream region. To test whether production of Fur is regulated by iron in *C. burnetii*, we examined gene expression at the transcriptional level. The transcriptional profile did not demonstrate any significant expression-level changes, suggesting that transcription of *C. burnetii* Fur is not responsive to environmental iron levels.

We then identified putative members of the *C. burnetii* Fur regulon. Initially, the FURTA was used to identify Fur boxes in a *C. burnetii* genomic library. One limitation of this approach is the use of *E. coli* Fur to identify putative *C. burnetii* Fur boxes. However, this method has been used successfully for other gram-negative bacteria as an initial screen (7, 51, 61) and for *Chlamydia trachomatis* (51). Also, *C. trachomatis* has a more divergent Fur, which shares 37% sequence similarity with the *E. coli* protein, compared to the 77% similarity that the *C. burnetii* protein shares with the *E. coli* protein (51). Unexpectedly, only two genes (CBU0970 and CBU1362) were predicted in the FURTA screen. Sequence analysis of CBU1362 revealed similarity to a family of cation efflux proteins (CDF proteins). However, transcriptional analysis did not predict iron regulation (Table 2). Since genes whose expression leads to a decrease in intracellular iron levels can show positive results in the FURTA assay (57), we theorized that CBU1362 is involved in iron efflux. Interestingly, Grass et al. recently described a novel role for ferrous iron export involving *yiiP* (encoding FieF), encoding a member of the CDF protein family, in *E. coli* (24). Since the acidity of the phagolysosome would favor the formation of ferrous iron over ferric iron, we hypothesized that CBU1362 would work in concert with iron acquisition proteins to modulate the level of intracellular ferrous iron.

CBU0970, designated *frg1* (*f*ur-*r*egulated *g*ene *1*), was also identified in the FURTA screen. Sequence analysis predicted a Fur box in the *frg1* promoter region with a 74% identity to the *E. coli* Fur box consensus sequence. However, BLAST analysis did not predict significant homology of the deduced amino acid sequence to any protein in the database. We theorize that Frg1 may play a role in extracellular iron binding due to a predicted signal sequence, a histidine-rich region, and two putative metal binding sequences. Since the FURTA is limited to identifying Fur boxes that are recognized by the *E. coli* Fur protein, we developed in silico approaches to identify other *C. burnetii* Fur-regulated genes.

One in silico approach was a screen of the TIGR genome annotation to identify iron acquisition homologues. The identification of a ferrous iron uptake system, *feoB*, is consistent with the lifestyle of *C. burnetii* in that the acidic environment would favor the formation of ferrous iron. Unexpectedly, other than a frameshifted ferripyrochelin gene (CBUA0035) and a single member of a known nonclassical siderophore transport system, *frgA* (CBU0421), no other homologues to described iron acquisition systems were identified in the *C. burnetii* genome. In contrast, the genome of the closely related bacterium *L. pneumophila* contains genes with homology to *feoB* (52), siderophore biosynthesis genes (1, 29, 37), genes encoding ferric reductases (49), and genes encoding a novel iron acquisition system that may utilize iron-loaded peptides (62). Of particular interest, the absence of TonB and proteins involved in classical siderophore transport suggest that ferric iron is not an important iron source for *C. burnetii*. However, the presence of a frameshifted ferripyrochelin gene (CBUA0035) and the presence of *frgA* (CBU0421), associated with nonclassical siderophore (37, 58) production, suggest that at an earlier stage of evolution, *C. burnetii* may have possessed acquisition systems for ferric iron. Recent studies on the *C. burnetii* genome suggest that this bacterium is in relatively early stage of genomic reduction, as an adaptation to its obligate intracellular lifestyle (55).

Despite the lack of annotated iron acquisition systems, a role for iron in the life cycle of the bacterium is suggested by the presence of members of the heme biosynthesis pathway (*hemH* [CBU0042], *hemE* [CBU0275], *hemF* [CBU1729], *hemD* [CBU2077], *hemY* [CBU2082], and a number of putative iron-containing proteins, including ferrodoxins [CBU0289, CBU0581, and CBU0598] and a superoxide dismutase containing iron [CBU1708]). Also of note is the absence of homologues of the highly conserved iron storage proteins ferritin and bacterioferritin. This suggests that *C. burnetii* uses another method to store ferrous iron to prevent the generation of toxic radicals. Perhaps, Fur also acts as a ferrous iron storage site in *C. burnetii*, similar to a suggested role in other bacteria (4).

The second in silico approach involved a bioinformatic survey of the genome by using the predicted Fur boxes of two *C. burnetii* genes (*feoB* [CBU1766] and *frg1* [CBU0970]) and the *E. coli* Fur box consensus sequence. Transcriptional analysis of these putative Fur-regulated genes was used to predict iron regulation compared to regulation of *C. burnetii feoB*. One limitation to this study was the incubation of *C. burnetii* under axenic conditions with a limited supply of nutrients to study changes in gene expression. However, this system did allow for tight regulation of iron levels. Using *feoB* as a strongly predicted Fur-regulated gene, we identified five additional genes (including *frg1* [CBU0970]) that demonstrated iron regulation. As expected, *feoB* (CBU1766; ferrous iron transport) and *frg1* (CBU0970; predicted iron binding protein-encoding gene) were strongly regulated by iron.

Unexpectedly, CBU1477 had a 4.5-fold increased induction in expression with a large standard deviation. CBU1477 was

annotated as *ahpC* (encoding alkyl hydroperoxide reductase), and it is a gene that may respond to oxidative stress. The large standard deviation in the observed induction was more likely due to a response to oxidative stress generated by the increased concentration of ferrous iron (50 μM) than due to iron regulation.

The predicted iron-regulated genes (*feoB* [CBU1766], *frg1* [CBU0970], CBU0216, CBU1106, and CBU0968) were further evaluated in a regulator/promoter two-plasmid system in concert with an *E. coli fur* deletion strain. A limitation in the interpretation of this study is the use of a heterologous system. However, this approach has been used successfully with *Vibrio anguillarum* to identify Fur-regulated genes (59). Another limitation is the use of a high-copy-number plasmid for the promoterless vector and a low-copy-number plasmid for the Fur vector. This may lead to a decreased level of Fur that may not sufficiently repress the promoters tested in this screen. However, a comparatively large repression of the *C. burnetii* Fur-regulated *feoB* gene was seen when *E. coli fur*, also on a low-copy-number plasmid, was transformed into the system as a control, suggesting that the experimental parameters used herein are adequate.

The *C. burnetii* and *E. coli* Fur regulation of the shared Fur box of CBU0970 and CBU0968 was not completely evaluated. As mentioned previously, the β-galactosidase activity expression levels were close to the background levels for the *frg1* (CBU0970) transformant. Though we predict weak Fur regulation for these genes, further direct analysis of Fur interaction with the putative Fur box via DNase footprinting or some other comparable method is needed to validate where *C. burnetii* Fur regulates these divergently transcribed genes.

Surprisingly, two of the genes predicted in the transcriptional screen (CBU0216 and CBU1106) did not demonstrate Fur regulation in the two-plasmid regulator/promoter system. These results predicted that these genes may belong to an iron regulon but are not regulated by Fur. Both CBU0216 and CBU1106 were annotated hypothetical proteins. Also, in the two-plasmid system, two genes not predicted to be iron regulated by transcriptional profiling, CBU0769, a predicted glutamine amidotransferase gene (class 1), and CBU0432, a predicted major facilitator family transporter gene, were strongly regulated by *E. coli* Fur and weakly regulated by *C. burnetii* Fur. This suggested that the transcriptional screen was limited to identifying strongly iron-regulated genes.

With only one predicted iron acquisition system (*feoB*) and few Fur-regulated genes, we hypothesized that iron may play a limited role in *C. burnetii* pathogenesis, compared to its closest relative *L. pneumophila* (12). To evaluate the role of iron in *C. burnetii* replication and, therefore, survival, J774A.1 murine macrophage-like cells were infected with *C. burnetii* Nine Mile phase II and then treated with an iron chelator, deferoxamine. Deferoxamine, a hydrophilic chelator, has been previously demonstrated to track to the lysosomal compartment (48) and used in experiments with J77A.1 cells (35). The observation of an increased replication rate of *C. burnetii* in the presence of an iron chelator was intriguing and differed from results reported by Howe and Mallavia (33). These results were supported in a preliminary L929 murine fibroblast cell line experiment (unpublished data). In addition, a preliminary experiment with the addition of iron to infected J774A.1 cells, the bacterial replication decreased (unpublished data). However, one limitation of the experiment by Howe and Mallavia was microscopic quantification of *C. burnetii* infection. Since that publication, a real-time PCR method to quantify the number of genome copies of bacteria during infection has been developed (10). Also, Howe and Mallavia noted that the uninfected J774A.1 cell line at 100 μM deferoxamine at 24 h had fewer cells than the cells incubated with 20 μM deferoxamine or left untreated (33), suggesting that 100 μM deferoxamine may have had a toxic effect on the host cell. Since the only change in *C. burnetii* replication was observed in the presence of 100 μM deferoxamine in that study, this result suggests that the reduced viability of the host cell mediated the reduced infection level and was not a consequence of iron-restricted bacterial replication.

The increase in replication rate under iron-chelated conditions suggests that *C. burnetii* prefers a low-iron environment. The paucity of potential iron acquisition and storage systems, compared to the amount in *L. pneumophila*, supports this notion. We speculate that the lack of high-affinity iron acquisition systems (i.e., TonB-dependent uptake systems) is the result of selection to maintain a low-intracellular-iron environment and also suggests that chelation of iron by the host, as a primary role in defense, would not be effective in controlling *C. burnetii* infection.

Another mechanism by which iron may contribute to host defense is through the generation of toxic oxygen radicals via the oxidative burst generated by gamma interferon-activated macrophages. The oxidative burst is generated via NADPH oxidase and consists of reactive oxygen intermediates, including superoxide anion and hydrogen peroxide (44). Once hydrogen peroxide is available, ferrous iron interacts with it in the Fenton reaction to produce the highly damaging hydroxyl radicals (44). Previously, Brennan et al. demonstrated that in NADPH oxidase-negative mice infected with *C. burnetii* phase I, splenomegaly was increased significantly compared to that in the control, suggesting that oxidative stress plays a role in controlling infection (9).

When we evaluated *C. burnetii* replication in iron-chelated cells containing different levels of expression of NADPH oxidase and, therefore, oxidative stress, replication increased in a decreased iron environment. Replication also increased in the J774D.9 cell line, which lacks clonal NADPH oxidase expression. This indicates that *C. burnetii* is also sensitive to other sources of iron-generated oxidative stress, such as in the aerobic environment of tissue culture.

With a significant increase in *C. burnetii* replication in the iron-chelated tissue culture model, bacterial infection was evaluated in the iron-overloaded and decreased-iron animal models. We postulated that the decrease in iron availability would modulate the level of oxidative stress and alter host response to infection. In the decreased-iron animal model, a significant increase in splenomegaly was observed. Since splenomegaly is an indirect measure of infection, we expected to see an increase in bacterial genome copy number. However, there was no significant change. In the iron overload model, as predicted, no significant difference in splenomegaly was observed. However, there was a significant decrease in genome copy number. This suggests the intriguing concept that in the iron-overloaded animal, the host may have an increased ability to control infection. A future experiment could be the determination

of the absolute level of iron required for *C. burnetii* survival. Since it would be difficult to decrease iron levels in an animal model, this nutrient requirement could be further evaluated in the acid activation buffer system. In this system, the iron levels can be tightly regulated, and metabolic processes could be assessed long-term in an extremely low-iron environment.

The lack of ferric transporters and other high-affinity iron acquisition homologues, in combination with an increased replication rate of *C. burnetii* in the presence of an iron chelator, predicts a preference for a decreased-iron environment. We predict that iron acquisition is less of a critical pathway for *C. burnetii* than it is for other pathogens and that a significant phenotype would not be observed in a *fur* knockout mutant. Therefore, *C. burnetii* has a functional Fur regulon with a limited set of genes, suggesting a minimal number of iron uptake systems and a low-iron environment enhance pathogenesis of the bacterium.

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