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Identification, Characterization, and Functional Analysis of a Gene Encoding the Ferric Uptake Regulation Protein in *Bartonella* Species

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Environmental iron concentrations coordinately regulate transcription of genes involved in iron acquisition and virulence via the ferric uptake regulation (*fur*) system. We identified and sequenced the *fur* gene and flanking regions of three *Bartonella* species. The most notable difference between *Bartonella* Fur and other Fur proteins was a substantially higher predicted isoelectric point. No promoter activity or Fur autoregulation was detected using a *gfp* reporter gene fused to the 204 nucleotides immediately upstream of the *Bartonella fur* gene. *Bartonella henselae fur* gene expression complemented a *Vibrio cholerae fur* mutant.

Bartonella, an extremely fastidious gram-negative bacillus, causes cat scratch disease, bacillary angiomatosis, and other syndromes (2, 13, 15, 29). Few data exist regarding the pathogenic mechanisms of this hemophilic bacterium (5), which can occupy two alternate niches: the iron-rich gut of obligately hematophagous arthropods and the iron-restricted bloodstream of mammals (11, 32). Acquisition of iron and expression of many virulence factors are under transcriptional regulation by the *fur* gene product, the ferric uptake regulation (Fur) protein, and its homodimeric complex (7). At sufficient intracellular iron levels, the corepressors Fur and Fe^{2+} form an active Fur-Fe^{2+} complex that binds a consensus sequence ("iron box," a 19-bp hyphenated dyad repeat [22] or three repeats of 6 bp of the sequence NAT[A,T]AT [7]) in the promoter region of genes regulated by Fur, down-regulating genes encoding iron-scavenging proteins (7, 22). We hypothesized that *Bartonella* species possess a *fur* gene homolog with a gene regulatory system influenced by iron levels.

Bacterial strains. Strains and plasmids used in this study are listed in Table 1. All *Bartonella* strains were used at low passage numbers (passes 1 through 3). *B. henselae* and *B. quintana* strains were grown on fresh chocolate agar (14), which provided a replete iron source. *B. bacilliformis* was grown on fresh heart infusion agar supplemented with 5% defibrinated rabbit blood (Hemostat Labs, Dixon, Calif.). Plates were incubated at 34°C (*B. henselae* and *B. quintana*) or 29°C (*B. bacilliformis*) in an enriched CO_2 environment for 5 to 7 days. Iron availability to *B. henselae* and *B. quintana* was restricted by adding the ferric-specific chelating agent EDDHA (ethylene diamine dihydroxy-*o*-phenylacetic acid) (The Complete Green Company, El Segundo, Calif.) (25) or by decreasing the hemoglobin (Hb) concentration in agar.

Vibrio strains were grown overnight in Luria-Bertani medium with the appropriate selective antibiotic(s), with or without the iron chelator 2,2-dipyridyl (Sigma-Aldrich, Inc., St. Louis, Mo.), as previously described (8). Because of limited growth at 37°C by CML13(pSYP4), all strains were grown at 30°C and then incubated at 37°C for 60 to 90 min before assays. Selective antibiotics were added to growth media as required, at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 25 μg/ml; kanamycin, 50 μg/ml; streptomycin, 100 μg/ml; tetracycline, 25 μg/ml.

Identification and analysis of *Bartonella* Fur. A 94-bp internal fragment of the *B. henselae fur* gene was amplified from *Bartonella* genomic DNA using degenerate primers designed from highly conserved *fur* gene sequences of other bacteria: FURMID-5′R, 5′-GGA ATT CCA (C,T)CA (C,T)GA (C,T) CA (C,T)(A,C)T (A,C,G,T)AT (A,C,T)GA-3′; FUREND-3′B, 5′-GGG ATC C(G,A)T A(A,C,G,T)A (G,A)(C,T)T C(A,C,G,T)A (G,A)(A,C,G,T)C G(G,A)T G-3′ (Operon Technologies Inc., Alameda, Calif.). The *B. henselae fur* gene was identified by probing a *B. henselae* genomic DNA library with the *B. henselae fur* gene fragment.

To generate *fur* gene fragment probes for screening of *B. quintana* and *B. bacilliformis* genomic DNA libraries, the *fur* gene open reading frame (ORF) was amplified by PCR from *B. quintana* and *B. bacilliformis* genomic DNA. The amplified *fur* gene fragments were then used to probe *B. quintana* or *B. bacilliformis* genomic DNA libraries. An ORF of 417 bp was identified for all three *Bartonella* species.

The deduced amino acid sequences of the *Bartonella* Fur proteins (Fig. 1) revealed that they each have a predicted length of 138 amino acids and are highly homologous (*B. henselae* and *B. quintana*, 89% identity; *B. henselae* and *B. bacilliformis*, 82% identity; *B. bacilliformis* and *B. quintana*,

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Bartonellae		
JK9R	*B. henselae*; human isolate from cutaneous bacillary angiomatosis lesion	J. E. Koehler
JK31	*B. quintana*; human isolate from cutaneous bacillary angiomatosis lesion	J. E. Koehler
BBL	*B. bacilliformis*; human isolate from blood	Generous gift of Robert Gilman, Lima, Peru
V. cholerae		
MBG40	O395 *irgA*::Tn*phoA* Sm^r Km^r	20
CML13	O395 *irgA*::Tn*phoA fur*::pCML13 Sm^r Km^r Ap^r	20
Plasmids		
pANT3	*ori*RSF1010 *mob*$^+$ Km^r Sm^r; promoterless *gfpmut3*	18
pSYP1	pANT3 backbone; putative *Bartonella fur* promoter upstream from *gfpmut3*	This study
pSYP2	pANT3 backbone; inverted putative *Bartonella fur* promoter upstream from *gfpmut3*	This study
pANT4	*ori*RSF1010 *bla*$^+$ *mob*$^+$ *lacI*^{q}::Km^r; *ptac* promoter upstream from *gfpmut3*	18
pSYP3	pANT4 backbone; *ptac* promoter upstream from *B. henselae fur*	This study
pACYC184	*ori*p15A Cm^r Tc^r	New England BioLabs, Inc., Beverly, Mass.
pSYP4	pACYC184 backbone; *ptac* promoter upstream from *B. henselae fur* cloned in place of *tet* gene	This study

79% identity). The *B. henselae* Fur protein had 38% amino acid identity with *Escherichia coli* and *Vibrio cholerae* Fur and 68% identity with *Brucella abortus* Fur. The *Bartonella* Fur amino acid sequence is rich in histidine residues, as are other Fur proteins (3, 4, 26), and 6 of the 42 invariant residues shown in Fig. 1 are histidines. Histidine is the primary amino acid involved in the binding of iron in heme (26, 28). The three *Bartonella* Fur proteins contain His-His-Asp-His, part of another suggested iron-binding motif, His-His-His-X-His-X_2-Cys-X_2-Cys, located at positions 86 to 96 of the *E. coli* and *V. cholerae* Fur proteins (17). The theoretical isoelectric point of *Bartonella* Fur is higher (8.1) than that of other Fur proteins (*Brucella abortus*, 6.1; *V. cholerae*, 5.5; *E. coli*, 5.7).

Confirmation of *Bartonella fur* genes by Southern blot analysis. *Bartonella* genomic DNA (approximately 2 μg each) was digested with *Hin*dIII and probed with approximately 250 ng of gel-purified, 417-bp *B. henselae fur* gene ORF. The *B. henselae fur* gene probe hybridized to a single band in each lane (in proportion to the homology with the *B. henselae fur* probe [Fig. 2]), indicating that each species likely contains a single *fur* gene, as in other bacterial species (1, 19, 21, 30, 31).

Complementation studies. Functional homology of *Bartonella* Fur with the Fur proteins of other gram-negative bac-

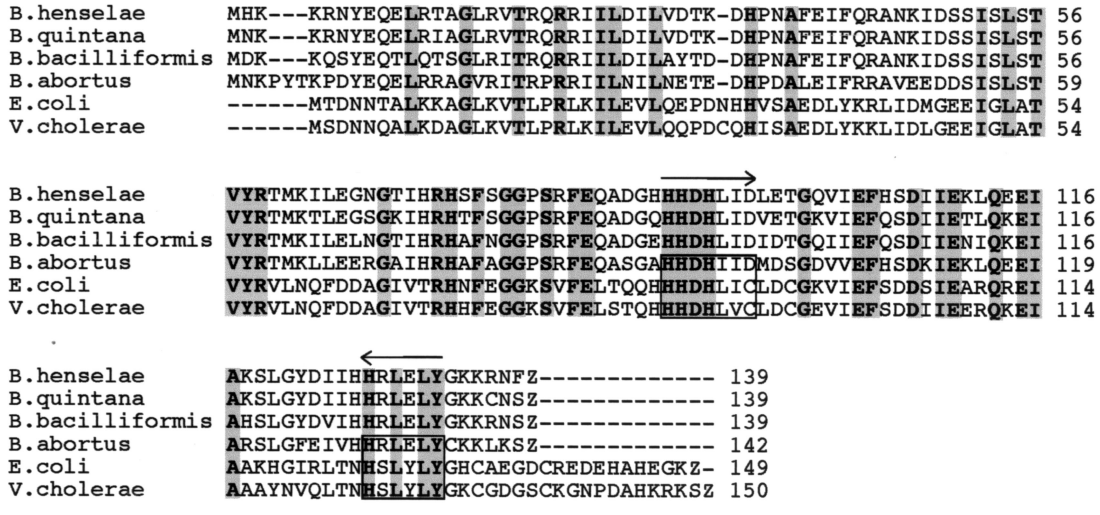

FIG. 1. Alignment of *Bartonella* species Fur proteins with those of other gram-negative bacteria. The Fur amino acid sequences of three *Bartonella* species and three other gram-negative bacterial species were aligned using the Clustal W program from the European Bioinformatics Institute (Cambridge, United Kingdom). There are 42 conserved amino acid positions (shaded). Degenerate oligonucleotide primers (arrows), based on two of the conserved regions (boxes), were designed and utilized in the initial amplification of a *B. henselae fur* gene fragment. Compared with other gram-negative species, *B. henselae* Fur had the greatest homology with *Brucella abortus*.

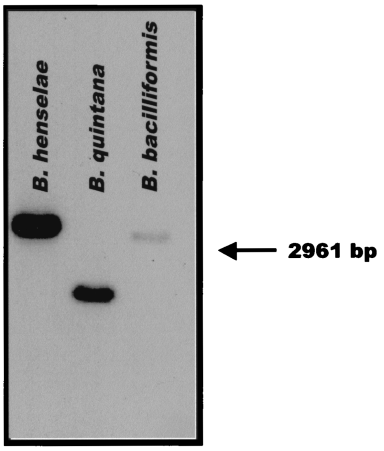

FIG. 2. Southern hybridization analysis of *Bartonella* species genomic DNA using a *B. henselae fur* gene probe. *B. henselae, B. quintana*, and *B. bacilliformis* DNAs were digested with *Hin*dIII. The fragments were separated in a 0.8% agarose gel, transferred to a nylon membrane, and probed with the 417-bp *B. henselae fur* gene. There was a single band in each lane of *Hin*dIII-digested genomic DNA: an approximately 4,200-bp fragment of *B. henselae* DNA, an approximately 2,000-bp fragment of *B. quintana* DNA, and an approximately 3,600-bp fragment of *B. bacilliformis* DNA.

teria was confirmed by complementation studies with a *V. cholerae fur* mutant. For these studies, the *B. henselae fur* gene was cloned into pACYC184. The plasmid pSYP3, in which the *B. henselae fur* ORF replaces the *gfp* cassette at the *Bam*HI and *Sph*I sites downstream from a constitutive *ptac* promoter in the pANT4 plasmid, was used as template to amplify the *ptac* promoter-*B. henselae fur* ORF sequence. The *ptac-fur* ORF was ligated into pACYC184, creating pSYP4. Clones were selected for chloramphenicol resistance and tetracycline sensitivity and confirmed by sequence analysis. pSYP4 and pACYC184 were introduced into *V. cholerae* strains. Expression of the *Bartonella fur* gene in the transformed *V. cholerae fur* mutant, CML13(pSYP4), was confirmed by separating proteins from whole-cell lysates by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown) (16).

The *V. cholerae* strain MBG40 has Tn*phoA* inserted into the iron-regulated *irgA* gene in the chromosome such that alkaline phosphatase reporter activity, determined by spectrophotometric measurement of hydrolysis of *p*-nitrophenyl phosphate by permeabilized cells (8, 19, 20), is strongly regulated by iron and Fur. In a *fur* mutant of this strain, CML13, alkaline phosphatase activity is not repressed by Fur despite changes in iron concentration (20). The ability of the *Bartonella fur* gene product to complement the *V. cholerae fur* mutant was studied by expressing *Bartonella fur* in this *Vibrio* strain, CML13(pSYP4), and determining alkaline phosphatase activity. Experiments were performed in triplicate, on four different days. Results were stratified by day and analyzed using a two-sided Mann-Whitney U test.

We observed that the iron-mediated regulation of reporter gene expression could be complemented by constitutive expression of the *B. henselae fur* gene in the *V. cholerae fur* mutant (Table 2). As shown previously (20), we found that the *V. cholerae* strain MBG40, producing a wild-type *V. cholerae* Fur protein, repressed reporter gene expression when grown on high-iron medium (induction ratio 56 [Table 2]). In contrast, the *V. cholerae fur* mutant CML13 had partially derepressed alkaline phosphatase reporter gene expression in the presence of high iron concentrations (induction ratio 10 [Table 2]). Plasmid pSYP4 complemented the *fur* mutation in strain CML13, restoring iron regulation very nearly to the level of strain MBG40. Repression of alkaline phosphatase activity may not have been complete because of the limited amino acid homology between *V. cholerae* Fur and *B. henselae* Fur (38%), the difference in pI, or both, which may result in reduced affinity of the *Bartonella* Fur-Fe^{2+} homodimer for the *Vibrio* Fur binding sequence. The absence of wild-type *Vibrio* Fur in CML13, CML13(pACYC184), and CML13(pSYP4) was confirmed by immunoblot analysis with polyclonal anti-*Vibrio* Fur antibody (data not shown). For reasons that are not clear, the vector plasmid pACYC184 partially restored iron regulation in strain CML13; nevertheless, the level of alkaline phosphatase expression in CML13(pACYC184) in high-iron media remained significantly higher ($P < 0.001$) than in either CML13(pSYP4) or MBG40. When iron was depleted, all four strains demonstrated derepression of reporter gene activity, although derepression was greatest in the *V. cholerae fur* mutant strain, a finding consistent with those of Litwin and Calderwood (20). Interestingly, the *V. cholerae fur* mutant CML13 did not produce equivalent alkaline phosphatase activity when grown under iron-replete compared with iron-depleted conditions. There was apparently some degree of regulation mediated by iron despite the absence of a functional Fur protein, as previously noted (20). Although the *Bartonella* Fur protein functionally complemented a *V. cholerae fur* mutant, there may be substantial structural differences compared with *V. cholerae* Fur. This is corroborated by the failure of a

TABLE 2. Complementation of a *V. cholerae fur* mutant by *Bartonella fur*

V. cholerae strain	Alkaline phosphatase activity (U/OD_{600} unit)[a]				Induction ratio[b]
	High-iron medium		Low-iron medium		
	Median	Range	Median	Range	
MBG40	16	12–20	889	750–1,339	56
CML13	119	82–179	1,200	1022–2,099	10
CML13(pACYC184)	34	23–42	1,022	748–1,256	30
CML13(pSYP4)	17	10–23	881	502–1,272	52

[a] Data were generated from triplicate experiments performed on four separate days. Data were stratified by day and analyzed using a two-sided Mann-Whitney U test. All comparisons were statistically significant ($P < 0.001$) except CML13(pSYP4) versus MBG40 ($P = 0.66$).

[b] Induction ratio = $\text{median}_{\text{low Fe}}/\text{median}_{\text{high Fe}}$.

polyclonal anti-*V. cholerae* Fur antibody (33) to recognize *Bartonella* Fur (wild type or overexpressed) by immunoblotting (data not shown).

Studies of the *fur* upstream region fused to a *gfp* reporter gene. The 204-bp region immediately upstream of the *B. henselae fur* gene ORF was amplified from genomic DNA and cloned into pANT3 just upstream of the *gfp* gene to create the plasmids pSYP1 (forward *fur* upstream sequence) and pSYP2 (inverted *fur* upstream sequence).

Plasmids were transferred into *Bartonella* strains by conjugation. *B. henselae*(pSYP1) and *B. quintana*(pSYP1) were grown on agar containing different concentrations of available iron and then scraped and suspended in phosphate-buffered saline for FACS analysis (FACScalibur; Becton Dickinson, Franklin Lakes, N.J.) (18). Chocolate plates were prepared with final EDDHA concentrations of 250, 300, 325, and 350 μM or with final Hb concentrations of 0.5, 1, 10, 20, and 30 mg/ml. (*B. henselae* growth is restricted on chocolate agar containing ≥400 μM EDDHA, and *B. quintana* growth is restricted at ≥350 μM EDDHA. Growth of both species is restricted at Hb concentrations of ≤1 mg/ml, and growth is optimal on chocolate agar plates containing 10 mg of Hb/ml.) The wild-type *B. henselae* and *B. quintana* strains were streaked on chocolate agar plates containing 10 mg of Hb/ml as negative fluorescence controls. *Bartonella* species containing a plasmid with a constitutively expressed *gfp* reporter gene (pANT4) or containing the upstream *fur* region in an inverted position (pSYP2) also were grown on agar containing different concentrations of available iron.

B. quintana(pSYP1), *B. henselae*(pSYP1), and the control *B. henselae*(pSYP2) demonstrated no change in GFP (green fluorescent protein) expression regardless of whether they were grown on iron-replete or iron-deficient medium. Wild-type *B. henselae* exhibited a small amount of autofluorescence, and *B. henselae*(pANT4) demonstrated stable fluorescence at an average of 1,000-fold higher than wild-type strains. *B. henselae* (pSYP2) and *B. quintana*(pSYP2) did demonstrate a small amount of GFP activity, but this activity did not vary with changes in iron concentration (data not shown) and probably represents artifactual activity.

Studies of other bacteria, e.g., *E. coli* (6, 7, 22), indicate that *fur* transcription is autoregulated in the presence of iron. We were unable to identify a Fur binding sequence or "iron box" that fulfilled the consensus of a 19-bp hyphenated dyad repeat (22) or two directed and one inverted 6-bp repeats (7) in the 204-bp region upstream of the *Bartonella fur* gene. However, there is one 19-bp sequence (positions 83 to 101) that conserves all four of the invariant nucleotides of the consensus (positions 6 and 14 to 16) but not the dyad repeat structure of the 19-bp sequence. This sequence is located within an ORF immediately upstream of *fur*, and functional, iron-regulated binding of this DNA sequence by *Bartonella* Fur was not detected by our *gfp* reporter assay. An autoregulatory binding sequence also is absent in the regions upstream of *fur* in *Bradyrhizobium japonicum* (9) and *V. cholerae* (19).

For other gram-negative bacteria, a promoter is located directly upstream of the *fur* gene; for some bacteria, a Fur binding region is located within the promoter region. The *E. coli fur* gene can be transcribed and regulated from its own promoter, or, in response to oxidative stress, it can be transcribed as part of an operon including the upstream *fldA* gene in addition to the downstream *fur* gene (35). We were unable to detect any promoter activity (regardless of iron availability) or autoregulatory activity in the 204-bp region upstream of the *Bartonella fur* gene. Because there are fewer than 35 bp between the upstream ORF and the *Bartonella fur* gene, and no promoter activity was detected in the upstream 204-bp region, it is possible that the *Bartonella fur* gene lacks its own promoter and that it is transcribed exclusively as part of an operon, which would be an unusual arrangement.

Conclusions. *Bartonella* has an obligate heme requirement (27, 34) and alternates between iron-rich and iron-restricted environments. In this study, we identified, cloned, and sequenced a *fur* gene in *B. henselae, B. quintana*, and *B. bacilliformis*. The deduced amino acid sequences of the three *Bartonella fur* genes demonstrate a high degree of homology among each other and substantial homology with the closest phylogenetic relative, *Brucella abortus* (24). To date, multiple attempts to generate a *Bartonella fur* mutant have been unsuccessful. Loss of the *fur* gene may be a lethal mutation in *Bartonella*, as occurs in some other gram-negative bacteria (1, 9, 23). Study of gene regulation by Fur in *Bartonella* will be critical to elucidating the mechanisms of iron acquisition, virulence gene expression, and *Bartonella* pathogenesis.

Nucleotide sequence accession numbers. The *fur* gene sequences of the three *Bartonella* species used in this study have been deposited in GenBank under accession numbers AF388196 (*B. henselae fur*), AF388197 (*B. quintana fur*), and AF388198 (*B. bacilliformis fur*).

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