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Transcriptional Control of the *rhuIR-bhuRSTUV* Heme Acquisition Locus in *Bordetella avium*

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Iron (Fe) is an essential nutrient for most bacterial pathogens. In these organisms, a variety of regulatory systems that respond to specific Fe complexes found within their vertebrate hosts have evolved. In *Bordetella avium*, the heme utilization locus encoded by *rhuIR-bhuRSTUV* mediates efficient acquisition of Fe from heme and hemoproteins. Control of *bhuRSTUV* expression is promulgated at two levels. When Fe is abundant, expression is repressed in a Fur-dependent manner which is partially relieved when Fe is limiting. In the presence of heme or hemoproteins, expression of the *bhuRSTUV* operon is induced via a three-component signal transduction cascade composed of RhuI, RhuR, and BhuR. Herein, we report the identification of two promoters (P_{rhuI} and P_{bhuR}) that control expression of the *rhuIR-bhuRSTUV* cluster. Primer extension analysis identified the transcriptional start site of P_{rhuI} within a putative Fur box. Transcriptional initiation of P_{bhuR} mapped within the *rhuR-bhuR* intergenic region. Maximal transcription from P_{bhuR} required Fe-limiting conditions, the presence of heme (or hemoglobin), and *rhuI*; however, analysis of transcripts produced from the *rhuIR-bhuRSTUV* locus revealed a pattern of low-level *bhuR* transcription in the absence of heme which originated from both P_{bhuR} and P_{rhuI}. Transcription from P_{rhuI} was repressed by Fe in the presence of *fur* and somewhat enhanced by the addition of hemin to Fe-limited media. The nature of this hemin-associated P_{rhuI} stimulation was *rhuI* independent and therefore not induced by heme via the BhuR-RhuR-RhuI signal cascade. Fe also repressed transcription from P_{bhuR} in a *fur*-dependent manner; however, activation from this promoter, in the presence or absence of heme, did not occur without *rhuI*.

Iron (Fe), an essential element for most pathogenic bacteria, must be acquired from the infected host. Within that host, however, Fe is usually sequestered within high-affinity ferromolecules such as transferrin, lactoferrin, and ferritin. Sequestration by these molecules maintains the free-Fe concentration in the tissue and fluids of vertebrates below 10^{-18} M (30), a level at which the growth of most bacteria is inhibited. To survive in these extremely Fe-limited host environments, bacteria express high-affinity Fe acquisition systems (reviewed in reference 19). In most cases, these acquisition systems are modulated in response to the availability of Fe in the local environment. Global Fe-dependent regulation is achieved primarily by Fur, a repressor protein whose regulation is dependent upon intracellular Fe concentration. Fur, which is expressed by a wide range of both gram-positive and gram-negative bacteria, has the capacity to reversibly bind Fe. In its repressor mode, Fe-bound Fur has affinity for the Fur box, a consensus motif that is located within or near Fur-regulated promoters (7). Binding of Fe-Fur to the Fur box is believed to interfere with binding of RNA polymerase (RNAP) to the promoter. Alternatively, binding of Fur might inhibit promoter escape of RNAP once the polymerase is bound to the promoter (10). When intracellular Fe levels decline to critical levels, the intracellular pool of Fur is no longer saturated with Fe. Since apo-Fur has little affinity for Fur boxes, Fur-dependent promoters are subsequently released from repression (10).

Successful pathogens have evolved a variety of mechanisms to acquire one or more of the biological Fe sources which are expressed by vertebrates (e.g., transferrin, ferritin, heme, hemoglobin, etc.) (3). Control of these systems solely by Fur would likely be a wasteful expenditure of resources, as it is unlikely that invading or colonizing bacteria will simultaneously encounter all of these sources of Fe in a single microhabitat. Thus, bacteria evolved additional regulatory elements that coordinately interact with Fur to rapidly and specifically respond to the presence of specific Fe-containing molecules. Various Fur-augmented regulatory systems have been described previously: siderophore-responsive transcription is dependent on AraC-type regulators in both *Pseudomonas aeruginosa* (14) and *Bordetella* spp. (2), LysR-type regulators have been implicated in the heme-responsive gene expression of *Vibrio vulnificus* (20) and *Bradyrhizobium japonicum* (12), and classical two-component sensor-kinase pairs have been described for *Corynebacterium diphtheriae* (25) and *Pseudomonas aeruginosa* (6). Sigma (σ) factors, particularly those denoted as extracytoplasmic function (ECF) σ factors, also participate in Fur-dependent gene regulation. In *Pseudomonas putida* (18), *Escherichia coli* (29), *Bordetella pertussis* (27), and *Bordetella avium* (17), specific Fe acquisition systems are maximally expressed only during Fe-limited growth in the presence of the cognate stimulatory ligand which, by binding to an extracellular receptor, stimulates the activity of the ECF σ factor for an associated ECF-dependent promoter.

ECF σ factor-based control of gene expression has been most extensively described in *E. coli*. In that bacterium, uptake

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of extracellular ferric dicitrate is governed by *fecIRABCDE* which is transcribed only when the bacterium is simultaneously stressed for Fe and ferric dicitrate is locally available. Responsiveness of *E. coli* to ferric dicitrate is mediated by the interaction of a three-component ECF σ regulatory cascade that includes FecA, the outer membrane receptor for ferric dicitrate (13); FecR, an inner membrane regulatory protein (23); and FecI, an ECF σ factor (1). Mechanistically, when FecA binds ferric dicitrate, a signal is transduced across the periplasm to FecR which transmits the signal to FecI (9). Acquisition of the signal by FecI elicits a change in its activation state which stimulates association of FecI to core RNAP. Recruitment of RNAP holoenzyme to P_{fecA} initiates direct transcription of the *fecABCDE* operon (1). Examination of the transcripts produced from the *fecIRABCDE* locus revealed two major mRNAs: a 1.5-kb mRNA corresponding to *fecIR* and a 2.5-kb mRNA corresponding to *fecA*. It has been proposed that the 2.5-kb *fecA*-bearing transcript is likely a degradation product of a 6-kb polycistronic transcript that contains sequences derived from the *fecABCDE* genes (8). In the absence of ferric dicitrate, basal expression of *fecABCDE* in Fe-stressed cells is regulated by Fur (29).

Nevertheless, an interesting conundrum was evident in the regulation of FecA. FecA is a requisite component of the FecIR-regulatory cascade and thus is required as a receptor of ferric dicitrate for initiating the signal cascade. In fact, low levels of FecA were detected in the outer membrane of Fe-stressed *E. coli* in the absence of ferric dicitrate. How, then, is FecA expressed in the absence of the stimulatory ligand? The answer to this question is yet to be revealed for *E. coli*. A mechanism to explain this conundrum, however, was recently described for *B. pertussis* (28). In *B. pertussis*, two promoters, P_{hurI} and P_{bhuR}, control expression of the heme utilization gene cluster *hurIR-bhuRSTUV*. Transcription from P_{hurI}, located upstream from *hurI*, is Fe regulated and heme independent. Transcription from P_{bhuR}, a promoter located within the *hurR-bhuR* intergenic region, is also Fe regulated and is induced by heme via HurI, the cognate ECF σ factor (27). Under Fe-stressed conditions in the absence of heme, expression of *bhuR* arises via transcriptional initiation at P_{hurI} in *B. pertussis*. Essentially, transcription does not terminate at the 3′ end of *hurIR* but proceeds infrequently into *bhuR* (28). This rare "readthrough" transcription is believed to be sufficient to express amounts of BhuR in the outer membrane for initial triggering of heme-dependent regulation of P_{bhuR} when heme becomes available.

B. avium, a gram-negative pathogen that infects domestic and wild fowl, elicits bordetellosis (or coryza), an upper respiratory illness defined by a loss of ciliated tracheal cells and a persistent, aggravated cough. The clinical presentation of coryza is highly similar to that of human pertussis (whooping cough) caused by taxonomically related *B. pertussis*. For that reason, *B. avium* has been used as a model for human pertussis. *B. avium* encodes the heme utilization locus *rhuIR-bhuRSTUV* (22), a heme uptake system with homology to *hurIR-bhuRSTUV* of *B. pertussis*. Expression levels of *rhuIR-bhuRSTUV* and *hurIR-bhuRSTUV* are regulated in similar manners in response to heme; i.e., in *B. avium*, expression of *bhuR* from P_{bhuR} is Fe regulated and heme responsive in an *rhuIR*-dependent manner (17). In this paper, the transcriptional architecture of the heme utilization locus in *B. avium* is described to compare and contrast the regulation of the heme uptake systems of *B. avium* and *B. pertussis*. The results confirm that unlike the regulation reported for *B. pertussis* (28), heme-independent expression of BhuR in *B. avium* is controlled by transcription which initiates at both the P_{rhuI} and P_{bhuR} promoters.

MATERIALS AND METHODS

Media, strains, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Strains of *B. avium* were maintained on brain heart infusion (BHI) agar, in BHI broth (Difco Laboratories, Detroit, Mich.), or in Chelex-treated complete defined medium (CDM) (5). Strains of *E. coli* were cultured on Luria-Bertani agar. For Fe-replete growth conditions, BHI or CDM was supplemented with 36 μM $FeSO_4$. Conditions of Fe limitation were achieved in CDM by reducing Fe by treatment with technical-grade Chelex-20 resin (Bio-Rad, Hercules, Calif.). Fe-limited conditions were achieved in BHI by supplementing the agar or broth with 100 μM ethylene-di-*o*-hydroxyphenylacetic acid (EDDHA). Unless otherwise noted, antibiotics used were as follows: ampicillin (200 μg/ml), rifampin (10 μg/ml), streptomycin (200 μg/ml), spectinomycin (10 μg/ml), tetracycline (10 μg/ml), kanamycin (50 μg/ml), and gentamicin (10 μg/ml). Antibiotics were obtained from Sigma Biochemicals (St. Louis, Mo.) and Amresco (Solon, Ohio). Biochemical reagents were purchased from Life Technologies, Inc. (Frederick, Md.) and Sigma Biochemicals. Restriction enzymes and DNA-modifying enzymes were obtained from MBI Fermentas, Inc. (Hanover, Md.). Deionized water with an electrical resistance of >18 MΩ was used to prepare all solutions.

Isolation of *B. avium* RNA. Total RNA was prepared from cultured cells by using a method developed by Chauhan and O'Brian (4). Cells grown to an optical density at 600 nm (OD_{600}) of 0.4 to 0.6 were harvested by centrifugation at 4°C. Cell pellets from 12.5 ml of culture were resuspended in 600 μl of lysis buffer (10 mM NaCl, 10 mM Tris [pH 8], 5% sodium dodecyl sulfate, 200 μg of proteinase K per ml) and incubated at 37°C for 5 min. Three hundred microliters of 5 M NaCl was added to the lysed cells. Mixtures were incubated on ice for 10 min, after which the debris was removed by centrifugation at 4°C. RNA in the supernatant was precipitated by centrifugation after the addition of 3 volumes of ethanol and incubation at −80°C for 1 h. RNA was resuspended in 1 mM EDTA, extracted with acid phenol-chloroform, and reprecipitated with ethanol. Purified RNA was treated with DNase I in the presence of human placental RNase inhibitor to eliminate contaminating DNA.

Primer extension analysis. Primers APbhuR1 (5′-CCGCTGCGTGAGAACA GACGAAAAG-3′) and APrhuI1 (5′-GCTTTTTGCGCAGCCAGCCATTCA G-3′) were end labeled in a reaction mixture containing 10 pmol of primer, 1 mM spermidine, 4 μl of [γ-^{32}P]ATP (3,000 Ci/mmol, 10 μCi/ml), 10 U of T4 polynucleotide kinase, and 1× labeling buffer (MBI Fermentas). Reaction mixtures were incubated at 37°C for 30 min, and reactions were terminated by heating to 95°C for 2 min. Labeled primers were purified by using a Sephadex G-25 column according to the manufacturer's instructions (Roche, Indianapolis, Ind.). Samples containing 20 μg of total RNA were ethanol precipitated, and pellets were resuspended in mixtures containing 0.2 pmol of labeled primer and 1× Superscript II reverse transcriptase reaction buffer (Invitrogen, Carlsbad, Calif.). RNA was denatured by heating to 95°C for 1 min, and labeled primers were annealed to the RNA by cooling samples to 70°C for 5 min and then to 30°C over a 10-min time span. Reaction mixtures were supplemented with 100 mM dithiothreitol, 10 mM deoxynucleoside triphosphates (dNTPs), Superscript II reverse transcriptase, and 1× Superscript II reverse transcriptase reaction buffer (Invitrogen). Reaction mixtures were incubated at 45°C for 30 min, terminated by the addition of EDTA to 100 μM, extracted with phenol-chloroform, precipitated with ethanol, and resuspended in Tris-EDTA buffer. Extended primers were resolved on 8 M urea–6% sequencing-grade polyacrylamide. For sizing purposes, a ladder was prepared by sequencing an appropriate template using the same primer.

Sequencing ladder for primer extension analysis. A total of 3 μg of pAD3 was denatured with 0.2 N NaOH, neutralized with 2 M NH_4Ac, and ethanol precipitated. Pelleted DNA was resuspended with 0.5 pmol of primer (APbhuR1 or APrhuI1) in 1× Sequenase buffer (USB Corp., Cleveland, Ohio). Primer and denatured template were annealed by heating to 65°C for 2 min and cooled slowly to room temperature. Labeling and termination reactions were performed according to the manufacturer's instructions (USB Corp.).

Construction of 4169rifΔ*rhuI*. The *rhuI* open reading frame (ORF) was deleted from the chromosome of wild-type (wt) *B. avium* 4169rif using allelic exchange (16). Splicing of overlapping ends (SOEing) by PCR was used to construct the allelic exchange construct pAEK22.1. The 507 bp immediately

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source
Strains		
B. avium		
4169rif	Wild type	22
4169rifΔ*rhuI*	4169rif; chromosomal *rhuI* deletion	This study
4169rif*fur*	4169rif; *fur* interrupted by ptr5-1 integration	17
4169rifΩ	4169rif; chromosomal Ω cassette insertion at 3′ end of *rhuR*	This study
Pho20	4169rif; *bhuR*::Tn*phoA*	5
Pho20*fur*	Pho20; *fur* interrupted by ptr5-1 integration	17
Pho20Ω	Pho20; chromosomal Ω cassette insertion at 3′ end of *rhuR*	This study
Pho20Ω*fur*	Pho20*fur*; chromosomal Ω cassette insertion at 3′ end of *rhuR*	This study
E. coli		
DH5α	F^- Ωϕ80*lacZ*M15 Δ(*lacZYA-argF*)1169 *deoR recA1 endA1 phoA hsdR17* ($r_K^-m_K^+$) *supE44* λ^- *thi-1 gyrA96 relA1*	Life Technologies
DH5αF′ *tet*	DH5α transconjugant with F′ *proAB* lac^qZΔM15 Tn*10*(Tet^r)	Life Technologies
SM10λ*pir*	Conjugation helper strain; RP4 plasmid integrated into the chromosome	Life Technologies
Plasmids		
pBluescript KS	Cloning vector; Amp^r	Stratagene
pET21a	Protein expression vector; P_{T7}; Amp^r	Novagen
pCVD442tet	Allelic exchange vector; λ*pir* dependent; *sacB*; Amp^r	22
pAD3	pBluescript KS; 5′ terminus of *B. avium bhuR* and 7.4 kb of upstream sequence	22
pRK415	Broad-host-range expression vector; Tet^r	15
pERM1	pMECA; 3-kb EcoRI fragment of p1016	22
p1016	pCOS9; 45-kbp fragment containing *rhuIR-bhuRSTUV* from 4169rif chromosome	22
pDJM31	pRK415; *rhuR-bhuR* intergenic region	17
pDJM41	pURF047; *rhuR-bhuR* intergenic region	17
pERM26	pRK415; *rhuI* ORF from pFI-7	17
pERM29	pBluescript KS; *bhuR* riboprobe template at EcoRI, XbaI	22
pERM30	pBluescript KS; *bhuRS* riboprobe template at EcoRI, XbaI	22
pAEK31	pBluescript KS; P_{bhuR} riboprobe template at EcoRI, BamHI	This study
pAEK32	pBluescript KS; *rhuI* riboprobe template at XhoI, HindIII	This study
pAEK33	pBluescript KS; *rhuIR* riboprobe template at XhoI, HindIII	This study
pAEK22.2	pCVD442tet; *rhuI* deletion construct at XbaI, SacI	This study
pAEK22	pBluescript KS; *rhuI* deletion construct in XbaI, SacI	This study
pAEK26.1	pET21a; *rhuR* at NdeI, EcoRI	16
pAEK37.2	pET21a; *rhuR-bhuR* with engineered EcoRI site at 3′ end of *rhuR*	This study
pAEK37.2Ω	pET21a; *rhuR*-Ω-*bhuR*	This study
pAEK37.3	pCVD442tet; *rhuR*-Ω-*bhuR* at XbaI, SacI	This study
pHP45Ω	pBR322; omega interposon at EcoRI	24

upstream from the *rhuI* GTG start codon were PCR amplified by using primers ΔrhuI-a2 (5′-GCTCTAGATAGATGTGATGCGGTTCTT-3′ [the XbaI site is underlined]) and ΔrhuI-b2 (5′-ACGCCGGCTGCGCTCATTTTTTGCAAGAAATATAA-3′ [the underlined region is complementary to the 5′ end of *rhuR*]) (PCR conditions were as follows: the PCR mixture contained 1× ThermoPol buffer, 10% dimethyl sulfoxide [DMSO], 250 μM dNTPs, 400 nM each oligonucleotide primer, and 1 U of Vent polymerase [Invitrogen], and PCR was carried out at 94°C for 1 min, 45°C for 1 min, and 72°C for 1 min for 30 cycles). Primers ΔrhuI-c (5′-ATGAGCGCAGCCGGCGT-3′) and ΔrhuI-d (5′-AGGGAGCTCTCTATTTAGTAACAGGAATCATTTA-3′ [the SacI site is underlined]) were used to amplify the 944 bp located immediately downstream from the *rhuI* ORF beginning at the ATG start codon of *rhuR* (PCR conditions were as follows: the PCR mixture contained 1× PCRx buffer [Promega, Madison, Wis.], 10% DMSO, 150 μM dNTPs, 1 μM each oligonucleotide primer, and 1 to 10 U of *Taq* polymerase, and PCR was carried out at 94°C for 1 min, 45°C for 1 min, and 72°C for 1 min for 25 cycles). PCR products, purified using a GFX PCR DNA and gel band purification kit (Amersham Pharmacia Biotech, Inc., Piscataway, N.J.), were used as templates for a modified PCR SOEing reaction in which 1 μl of each PCR product was added to an annealing reaction mixture containing 1× ThermoPol buffer (Invitrogen), 10% DMSO, and 250 μM dNTPs. This mixture was heated to 95°C for 5 min and cooled to 25°C over a period of 3 min to allow the ends of each PCR product to anneal, thus generating partially double-stranded templates. The double-stranded templates were repaired by using 1 U of Klenow fragment (MBI Fermentas, Inc.). After 10 min at 25°C, Klenow fragment was heat inactivated by incubation at 95°C for 5 min. Primers (ΔrhuI-a2 and ΔrhuI-d, at a concentration of 1 μM each) and 1 U of Vent polymerase (Invitrogen) were added to the mixture to initiate PCR amplification (94°C for 1 min, 45°C for 1 min, 72°C for 3 min, 25 cycles). The PCR SOEing product was ligated into pBluescript KS(+) at the XbaI and SacI sites to engineer pAEK22. After the sequence of pAEK22 was confirmed by automated DNA sequencing, the insert was released from the plasmid by digestion with XbaI and SacI, gel purified, and ligated into pCVD442tet, which had been digested with the same enzymes. The resulting plasmid, pAEK22.1, was moved into *B. avium* 4169rif by conjugation (22). Transconjugants were selected for growth on BHI agar containing tetracycline. Cells that had undergone a second recombination event to remove the plasmid sequences were selected for growth on BHI agar containing 20% sucrose. Deletion of *rhuI* in 4169rifΔ*rhuI* was confirmed by colony PCR and Southern hybridization.

Construction of 4169rifΩ, Pho20Ω, and Pho20Ω*fur*. An omega (Ω) fragment containing bidirectional transcriptional and translational terminators was inserted directly downstream from the *rhuR* ORF in the chromosome of *B. avium* strains 4169rif, Pho20, and Pho20*fur* using allelic exchange. In brief, the plasmid pAEK37.3, used for allelic exchange, was constructed using the following cloning scheme. A 993-bp fragment containing the *rhuR-bhuR* intergenic region and a portion of the *bhuR* ORF was amplified from pERM1 by PCR using primers rhuRΩ (5′-GGAATTCGATTCCTGTTACTAAATAGATTCGTAAAAAC-3′ [the EcoRI site is underlined]) and bhuRΔD (5′-TCCGAGCTCCGCCTGAATCAACCATGTCGTGTTGT-3′ [the SacI site is underlined]) (PCR conditions were as follows: the PCR mixture contained 1× Fermentas buffer without Mg^{2+}, 1.5 mM $MgCl_2$, 10% DMSO, 250 μM dNTPs, 400 nM each oligonucleotide

primer, and 1U of *Taq* polymerase [MBI Fermentas], and PCR was carried out at 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min for 30 cycles). This fragment was cloned into the EcoRI- and SacI-digested plasmid pAEK26.1, which contains the *rhuR* ORF immediately upstream from an EcoRI site, to generate plasmid pAEK37.2. The Ω fragment from pHP45Ω was cloned into the EcoRI site of pAEK37.2 to engineer pAEK37.2Ω. This plasmid was digested with XbaI and SacI to isolate a fragment containing *rhuR*-Ω-*bhuR* which was inserted into the same sites of plasmid pCVD442tet to produce pAEK37.3. The construction of pAEK37.3 was confirmed by automated DNA sequencing. The plasmid was transformed into SM10λ*pir* and subsequently conjugated into *B. avium*. Transconjugants were selected for growth on BHI agar containing tetracycline. Cells that had undergone the second recombination event were selected for growth on BHI agar containing 20% sucrose. The mutant strains 4169rifΩ, Pho20Ω, and Pho20Ω*fur* were isolated, and their mutant genotypes were confirmed by Southern hybridization.

β-Galactosidase assay. Expression of the *lacZYA* reporter gene in pDJM41 was determined by measuring β-galactosidase activity (17). Briefly, cultures of Fe-limited cells grown overnight were used to inoculate secondary cultures. The secondary cultures were incubated at 37°C for 16 to 20 h. Bacteria in 1.0 ml of culture were pelleted by centrifugation for 5 min at 5,000 × *g*. Cells were resuspended in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 38 mM β-mercaptoethanol), and the OD_{600} of the cell suspension was adjusted to 0.28 to 0.70. After diluting 400 μl of the suspension with 400 μl of Z buffer, cells were permeabilized by the addition of 45 μl of 0.1% sodium dodecyl sulfate and 90 μl of chloroform. Cell suspensions were vortexed for 10 s, followed by incubation at 30°C for 15 min. Enzymatic reactions were initiated by the addition of 160 μl of a solution containing 4 mg of *o*-nitrophenyl-β-D-galactopyranoside per ml. Following incubation at 30°C, samples were observed for the development of a yellow color, at which point 400 μl of 1 M Na_2CO_3 was added to terminate the reaction. After a brief centrifugation to pellet debris and chloroform, the OD_{420} and OD_{550} of each reaction were recorded, and the relative β-galactosidase activity was calculated by use of the following formula (21): $\{1{,}000[OD_{420} - 1.75(OD_{550})]\}/(t)(0.4)(OD_{600})$, where t is the time of the reaction in minutes. Relative enzyme activities are reported as the means of triplicate assays.

Alkaline phosphatase assay. Expression of the *bhuR*::*phoA* fusion of *B. avium* mutants Pho20, Pho20Ω, Pho20*fur*, and Pho20Ω*fur* was determined by using a modified *p*-nitrophenyl phosphate assay (11). Cultures of Fe-limited cells grown overnight were used to inoculate secondary cultures in BHI broth under either Fe-replete (36 μM $FeSO_4$) or Fe-limited (100 μM EDDHA) conditions. At stationary phase, 1 ml of the culture was centrifuged, and the cell pellet was resuspended in a solution containing 10 mM Tris (pH 8) and 100 mM NaCl. Cell densities of the resuspensions were adjusted to an OD_{600} of 0.4 to 0.8. An aliquot containing 500 μl of the cell suspension was added to an equal volume of 1 M Tris (pH 8), and the reaction was initiated by the addition of 100 μl of 4% *p*-nitrophenyl phosphate. Following incubation at room temperature, samples were observed for development of a yellow color, at which point 100 μl of 1 M KH_2PO_4 was added to terminate the reaction. After a brief centrifugation to pellet debris, the OD_{420} and OD_{550} of each reaction were recorded, and the relative alkaline phosphatase activity was calculated by use of the following formula (21): $\{1{,}000[OD_{420} - 1.75(OD_{550})]\}/(t)(0.5)(OD_{600})$, where t is the time of the reaction in minutes. Relative enzyme activities are reported as the means of triplicate assays.

RNase protection assay (RPA). Construction of the DNA templates for the *bhuR* and *bhuRS* probes (pERM29 and pERM30, respectively) was described previously (22). PCR was used to construct the templates for the *rhuI* and *rhuIR* probes. Primers rhuIRPA1 (5′-CCGCTCGAGTCCATCGCTTCTATGCT-3′ [the XhoI site is underlined]) and rhuIRPA2 (5′-CCCAAGCTTACGGTAGT GATTGAGCA-3′ [the HindIII site is underlined]) amplified a 215-bp fragment within the *rhuI* ORF. A 254-bp fragment which contains the last 171 bp of *rhuI* and the first 86 bp of the *rhuR* 5′ end was amplified by primers IRRPA1 (5′-CCGCTCGAGTGTCTGCTCTGCCCC-3′ [the XhoI site is underlined]) and IRRPA2 (5′-CCCAAGCTTCTGCCCCGAAGCAAGA-3′ [the HindIII site is underlined]). For both primer sets, the PCR conditions were as follows: the PCR mixture contained 1× PCRx buffer (Promega), 10% DMSO, 100 μM dNTPs, 1 μM each oligonucleotide primer, and 1 to 10 U of *Taq* polymerase, and PCR was carried out at 95°C for 45 s, 45°C for 45 s, and 72°C for 60 s for 30 cycles. The resulting amplicons were ligated into pBluescript KS(−) at the XhoI and HindIII sites. The sequences of pAEK32, the *rhuI* probe template, and pAEK33, the *rhuIR* probe template, were confirmed by automated DNA sequencing. To construct the P_{bhuR} template, pAEK31, a 195-bp fragment, was released from pDJM31 by digestion with EcoRI and BamHI. This fragment, containing 31 bp of the 3′ end of *rhuR*, the entire 102-bp intergenic region, and 47 bp of the 5′ end of *bhuR*, was ligated into the EcoRI and BamHI sites of pBluescript KS(−).

Antisense RNA probes for RPAs were generated by using a MaxiScript in vitro transcription kit (Ambion Inc., Austin, Tex.). Plasmids pERM29 and pERM30 were linearized with XhoI and transcribed using T3 polymerase. Plasmids pAEK31, pAEK32, and pAEK33 were linearized with EcoRI and transcribed using T7 polymerase. The probes, generated by incorporating [α-^{32}P]CTP, were labeled according to the manufacturer's protocol, gel purified using an 8 M urea–5% polyacrylamide gel, and eluted from the gel at 37°C overnight using probe elution buffer (HybeSpeed RPA kit; Ambion). The activity of each probe was determined by using a Wallac (Turku, Finland) 1409 liquid scintillation counter. RPAs were performed by using a HybSpeed RPA kit (Ambion) according to manufacturer's protocol. A total of 15 μg of RNA was used in assays with the P_{bhuR}, *rhuI*, and *rhuIR* probes and 5 μg of RNA was used in assays with the *bhuR* and *bhuRS* probes. Control reactions, in which pAEK31 was linearized with BamHI and transcribed with T3 polymerase generating labeled sense-strand RNA, were used to ensure that protected fragments were not the products of contaminating DNA in the RNA samples. The final reactions were separated by electrophoresis through an 8 M urea–5% polyacrylamide gel. Gels were dried and exposed to blue sensitive autoradiographic film (Marsh Bioproducts, Rochester, N.Y.) at −80°C using an intensifying screen.

Nucleotide sequencing and analysis. Nucleotide sequencing was performed at the Biopolymer Facility at Roswell Park Cancer Institute (Buffalo, N.Y.). Sequence analysis was performed by using the Wisconsin Package version 9.0 (Genetics Computer Group, Madison, Wis.) and ClustalW (http://www.ebi.ac.uk/clustalw).

RESULTS

Primer extension analysis of P_{rhuI} and P_{bhuR}. As an initial step to define the transcriptional architecture of the *rhuIR-bhuRSTUV* (*rhu-bhu*) locus, the transcriptional start sites of P_{rhuI} and P_{bhuR} were identified by use of primer extension. For the extension reactions, total RNA was isolated from cultures of *B. avium* 4169rif which had been cultured in CDM under conditions of Fe limitation. For comparison, total RNA was also obtained from 4169rif cultured under Fe-replete conditions. To evaluate the capacity of the cells to respond to heme as a putative source of Fe and as an inducer of the *rhu-bhu* locus, total RNA was also isolated from cultures of Fe-starved 4169rif to which heme had been added. In those cultures, the broth was supplemented with EDDHA to remove any contaminating Fe that might have been present in the hemin stock solution.

Primer extension experiments revealed that transcription from P_{rhuI} was strongly repressed in Fe-replete cultures (Fig. 1A). In Fe-limiting growth, transcription from P_{rhuI} initiated from a G residue located 28 bases upstream from the *rhuI* start codon. Analysis of the region of DNA which contained this G residue showed that this transcriptional start site was located within a putative Fur box which is optimally spaced from −35 and −10 elements that are predicted to be σ^{70} dependent (17). These results indicated that transcription from P_{rhuI} is regulated by Fe, perhaps in a Fur-dependent manner. The observations were also consistent with those reported for *B. pertussis* (28). Unexpectedly, when hemin was the sole source of Fe, transcriptional initiation in 4169rif was significantly increased from P_{rhuI} and suggests that heme is an inducer of this promoter.

As was observed for P_{rhuI}, transcription from P_{bhuR} in cells obtained from Fe-replete cultures was not detected (Fig. 1B). Hemin supplementation elicited initiation of transcription at an A residue located 37 bases upstream from the *bhuR* start codon, a pattern of transcriptional initiation which was consis-

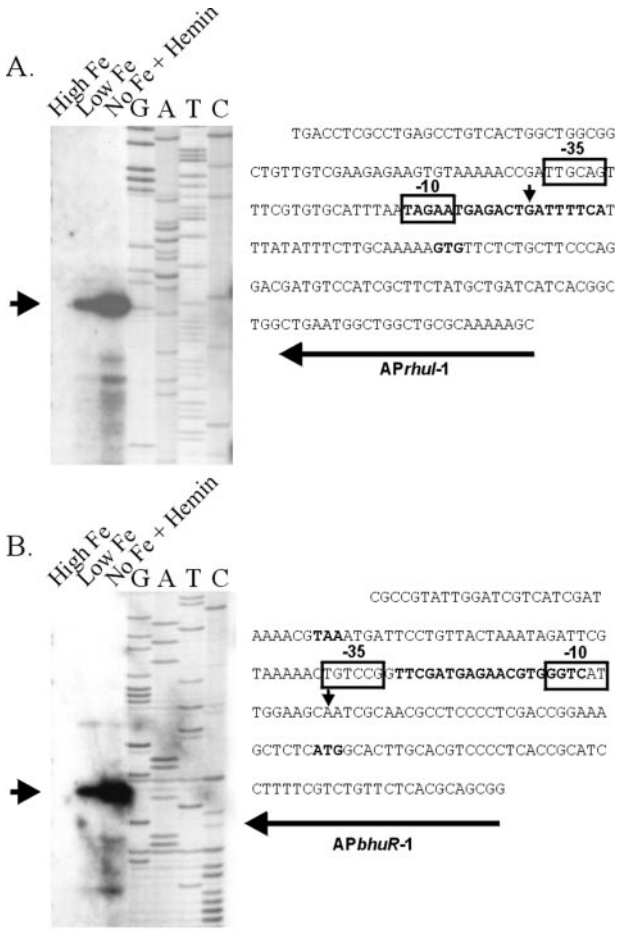

FIG. 1. Primer extension analysis shows an Fe-dependent, heme-independent P_{bhuR} activity. Primers (A) APrhuI-1 and (B) APbhuR-1 were annealed to total RNA isolated from 4169rif grown in CDM broth supplemented with 36 μM $FeSO_4$ (High Fe), no added Fe (Low Fe), or 300 μM EDDHA plus 1 μM hemin (No Fe+ hemin). Extended primer reactions and sequencing reactions were resolved in polyacrylamide-urea gels. The locations of primers used for nucleotide sequencing are designated in the nucleotide sequences. Putative −10 and −35 regions are boxed. Putative Fur boxes and start (ATG) and stop (TAA) codons are denoted in boldface type, while arrows indicate transcriptional start sites.

tent with previous experiments that demonstrated hemin-dependent expression and induction of *bhuR* (17). Surprisingly, when cells were cultured under Fe-limiting growth conditions in the absence of hemin, the level of transcription from P_{bhuR} in those cells was similar to the level of transcription from P_{rhuI} in cells cultured under equivalent conditions of Fe limitation. This pattern of transcription was in contrast to the pattern of transcriptional initiation reported for *B. pertussis* and *Bordetella bronchiseptica*. In those species, heme-independent *bhuR* expression originated solely from P_{hurI} (28).

To discount the possibility that P_{bhuR}-dependent extension products in the primer extension experiments were artifacts caused by a premature termination of the extension reactions generated by inhibitory secondary structures in the RNA located proximal to the putative start site, primer extension analysis was also performed using an artificial RNA substrate. RNA for this control experiment was obtained from in vitro transcription of pAEK31, a plasmid containing a DNA fragment encompassing the 3′ end of *rhuR*, the *rhuR-bhuR* intergenic region, and the 5′ end of *bhuR* cloned into pBluescript. When primer extension reactions were performed on this artificial RNA substrate, the extension reaction proceeded through the putative start site and into the *rhuR* sequences (data not shown). In brief, these results indicated that the start site revealed by the primer extension product for the P_{bhuR} site was not an artifact caused by premature termination of reverse transcriptase but is likely to be the actual initiation site for P_{bhuR} transcriptional initiation in vivo.

Comparison of the *B. avium rhuR-bhuR* and *B. pertussis hurR-bhuR* intergenic regions. Since *B. avium* and *B. pertussis* exhibited differences in P_{bhuR} transcriptional patterns, the nucleotide sequences of the regions surrounding these promoters in the two species were compared in an attempt to identify structural motifs which might mediate those differences. While the stop codon of the *rhuI* ORF and the start codon of *bhuR* are separated in *B. avium* by 105 bp of DNA, the two corresponding sites in *B. pertussis* are separated by 207 bp. Homology between these two intergenic regions is most apparent when the P_{bhuR} start site of *B. avium* and the P_{bhuR} start site of *B. pertussis* (28) are aligned. In this orientation, the putative −35 and −10 elements are identical. Seventy-two nucleotides (nt) in the *rhuR-bhuR* intergenic region of *B. pertussis*, which are located downstream from the *bhuR* transcriptional start site, are absent in the corresponding intergenic region of *B. avium*. Computer analysis of the 72-bp region of *B. pertussis* did not reveal known regulatory motifs. While experiments have yet to reveal a new regulatory structure within this segment of DNA, it is reasonable to hypothesize that the 72-nt region contributes to the apparent differences in heme-independent *bhuR* transcription and polypeptide expression observed between *B. avium* and *B. pertussis*.

***rhuI* contributes to heme-independent expression of *bhuR*.** When overexpressed from a plasmid in *B. avium*, recombinant *rhuI* induces both Fe-independent and heme-independent expression of *bhuR* (17). To further assess the role of *rhuI* on P_{bhuR} activity, a mutant of 4169rif which had a precise deletion of the *rhuI* ORF was engineered. Engineering of the *rhuI* mutant was such that the adjacent sequences encoding *rhuR* and the sequences encoding the *rhuR-bhuR* intergenic region were undisturbed. P_{bhuR} activity was monitored by measuring the activity of an extrachromosomal P_{bhuR}::*lacZYA* reporter in pDJM41 (Fig. 2A). Constitutive expression of recombinant *rhuI* in those strains was produced by introduction of pERM26, as needed. All strains were cultured in BHI broth under Fe-replete (36 μM $FeSO_4$), Fe-limited (100 μM EDDHA), or Fe-limited plus hemin (100 μM EDDHA plus 5 μM hemin) growth conditions.

Analysis of 4169rif(pDJM41, pRK415) cultured under Fe-replete growth conditions revealed only low-level expression of β-galactosidase activity which increased only twofold when cells were cultured in Fe-limited broth (Fig. 2B). Supplementation of the Fe-limited broth with hemin, however, enhanced β-galactosidase activity fivefold relative to Fe-replete conditions (Fig. 2B) (22), a pattern of expression consistent with prior reports which indicated that heme was a positive inducer of P_{bhuR} (17, 26). In comparison to P_{bhuR} activity in cells cul-

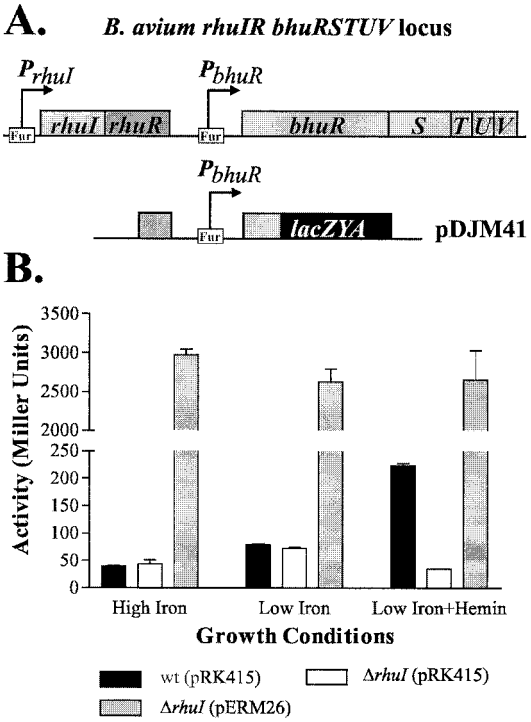

FIG. 2. Complementation of Δ*rhuI* mutant. (A) Schematic representations of the *rhuIR-bhuRSTUV* locus in the *B. avium* genome. Positions and orientations of P_{rhuI} and P_{bhuR} promoters (arrows), putative Fur boxes (open boxes), and open reading frames (shaded boxes) are indicated. pDJM41 encodes a 199-bp segment of DNA encompassing the *rhuR-bhuR* intergenic region (thick line) which is fused to *lacZYA* (shaded box) (not drawn to scale). (B) Both wt 4169rif and 4169rifΔ*rhuI* were cotransformed with pDJM41 and the expression vector pRK415 or pERM26, as appropriate. pERM26 harbors the *rhuI* ORF. Strains were cultured in BHI broth supplemented with 36 μM $FeSO_4$ (High Fe), 100 μM EDDHA (Low Fe), or 100 μM EDDHA plus 5 μM hemin (Low Fe+Hemin). β-Galactosidase activities were measured from log-phase cultures.

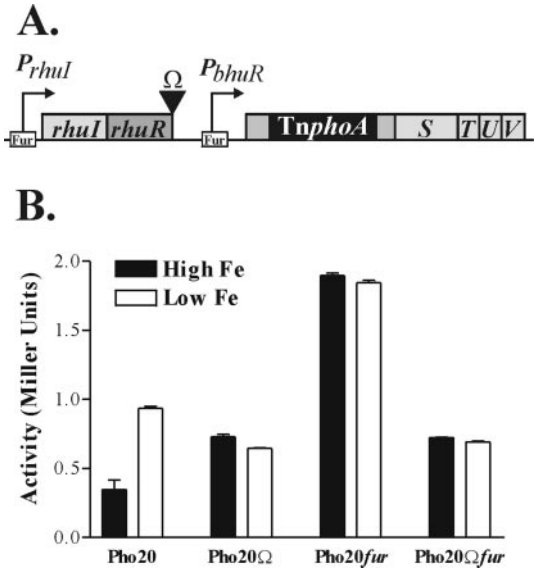

FIG. 3. Fe-dependent expression of *bhuR* from P_{rhuI} is regulated by Fur. (A) Schematic representation of alterations made to the *rhuIR-bhuRSTUV* locus in *B. avium* (not drawn to scale). Positions and orientations of P_{rhuI} and P_{bhuR} promoters (arrows), putative Fur boxes (open boxes), the site of Ω cassette insertion (solid triangle), open reading frames (shaded boxes), and the site of the insertion of Tn*phoA* are indicated. In strain Pho20, *bhuR* is interrupted by an in-frame insertion of *phoA.* Hence, *bhuR* expression was assessed by measuring alkaline phosphatase activity of cultured cells. Pho20*fur* contains an insertional mutation in *fur*. An Ω cassette was inserted immediately downstream from *rhuR* in both Pho20 and Pho20*fur* to generate Pho20Ω and Pho20Ω*fur*. (B) All strains were cultured in BHI broth supplemented with 36 μM $FeSO_4$ (High Fe) or 100 μM EDDHA (Low Fe), and alkaline phosphatase activity was measured from stationary-phase cultures.

tured under Fe-replete growth conditions, β-galactosidase activity of 4169rifΔ*rhuI*(pDJM41, pRK415) was also enhanced twofold when exposed to Fe limitation (Fig. 2B). Yet β-galactosidase activity of 4169rifΔ*rhuI*(pDJM41, pRK415) was not elevated when the Fe-limited culture broth was supplemented with hemin (Fig. 2B). Introduction of pERM26 into 4169rifΔ*rhuI*(pDJM41) highly enhanced β-galactosidase activity under all growth conditions (Fig. 2B).

These results indicated that *rhuI* was essential for heme-dependent activation of P_{bhuR}. Fe-dependent expression of *bhuR*, however, was independent of *rhuI* (17). Yet it was also clear from these experiments that overexpression of a recombinant *rhuI* constitutively activated P_{bhuR} in the absence of heme, which suggested that RhuI functions as a P_{bhuR}-dependent sigma factor in the absence of heme.

Assessing the role of P_{rhuI} on Fe-dependent, heme-independent *bhuR* expression. Fe-dependent, heme-independent expression of *bhuR* in *B. pertussis* and *B. bronchiseptica* is hypothesized to occur by readthrough transcription initiating from P_{hurI} (28). Thus, expression of *bhuR* in cells cultured under Fe-limited, heme-free growth conditions would be expected to be absent in a mutant in which transcription is terminated at *rhuR*. To test this hypothesis, various mutants were constructed in which an Ω cassette, a DNA cassette containing multiple translational and transcription terminators, was inserted into the chromosomes of 4169rif and Pho20 at a position 2 bp downstream of the TAA translational terminator of *rhuR* (5). Pho20 is a reporter mutant containing an in-frame *phoA* insertion into *bhuR* (Fig. 3A).

Using Pho20 and Pho20Ω, Fe-dependent expression of *bhuR* was assessed by measuring alkaline phosphatase activity of cultures grown under Fe-replete or Fe-limited conditions. As previously reported, alkaline phosphatase activity of Pho20 was low when cells were cultured under Fe-replete conditions and increased slightly when cells were cultured under Fe-limiting conditions (Fig. 3B). These data were consistent with prior reports (17) which demonstrated that *bhuR* expression responded to Fe-dependent repression. When the PhoA activity of Pho20Ω was evaluated, it was found that the level of alkaline phosphatase activity of Pho20Ω was similar to the alkaline phosphatase activity of Pho20 when the mutants were cultured under Fe-limiting growth conditions. Remarkably, the alkaline phosphatase activity of Pho20Ω was essentially unchanged, even when Pho20Ω was cultured under Fe-replete conditions (Fig. 3B). This unexpected result suggested that the Ω cassette likely imposed an artificial Fe-independent transcriptional activity that promoted expression of the *bhuR*::*phoA* fusion. It should be noted that similar problematical results were ob-

served when a mutant containing an Ω cassette was incorporated into the *hurR-bhuR* intergenic region of a plasmid-borne copy of the *B. pertussis hurIR-bhuR-lacZ* locus (28). In that case, introduction of the Ω cassette stimulated an increase in reporter activity when the cells were cultured under Fe-replete conditions. Thus, these data obtained in *B. avium* and in *B. pertussis* using Ω cassette mutants neither supported nor refuted the hypothesis that *bhuR* expression under conditions of Fe-limited growth was promulgated by readthrough transcription from P_{rhuI}. Rather, it is obvious that the Ω cassette is not a useful tool for experiments to artificially terminate transcription in either *B. avium* or *B. pertussis* and that results from such experiments need to be interpreted with care.

The role of Fur in expression of *bhuR*. Alkaline phosphatase activity of Pho20*fur*, a mutant of Pho20 in which the genomic copy of *fur* is genetically disrupted (17), was significantly higher than its Pho20 parent when cultured under Fe-replete conditions (Fig. 3B). These data supported a model of Fur regulation of Fe-dependent repression of *bhuR* (17). Under Fe-replete conditions, the alkaline phosphatase activity of Pho20*fur* was also significantly higher than the alkaline phosphatase activity of Pho20Ω (Fig. 3B). This pattern of expression of the reporter suggests that the artifactual problems inherent in Ω cassette mutants can be overcome by evaluating the effects of the Ω cassette on readthrough transcription in *fur* backgrounds in *B. avium*. Indeed, the results show that alkaline phosphatase activity of Pho20Ω*fur* was reduced compared to the activity of Pho20*fur* (Fig. 3B) and indicated that Fe-dependent and Fur-dependent activity from P_{rhuI} is at least partly responsible for *bhuR* expression.

Evaluation of P_{bhuR} transcriptional activity. Primer extension analysis indicated that transcription initiated from both P_{rhuI} and P_{bhuR} when cells were cultured under conditions of Fe limitation in the presence or absence of hemin (Fig. 1). From these data, it was inferred that P_{bhuR} is responsible for heme-independent expression of *bhuR*. These observations are in contrast to results observed for *B. pertussis* (28) in which P_{bhuR} activity was solely heme dependent. In the Pho20*fur* and Pho20Ω*fur* mutant strains, P_{rhuI} activity was at least partially responsible for Fe-dependent *bhuR* expression (Fig. 3B). To further analyze heme-independent expression of *bhuR* in *B. avium*, the effects of the *fur*, Δ*rhuI*, and Ω cassette mutations on P_{bhuR} activity were examined using primer extension as a direct measurement of transcriptional activity from that promoter.

Primer extension reactions using total RNA harvested from BHI cultures of 4169rif, 4169rifΔ*rhuI*, 4169rifΩ, and 4169rif*fur* revealed a previously unknown activity of RhuI. As previously reported, P_{bhuR} was inactive when 4169rif was cultured under conditions in which Fe was abundant (Fig. 1B and 4B). Under Fe-limiting conditions, however, significant transcription from P_{bhuR} was detected regardless of the presence or absence of hemin (Fig. 1B and 4B). Transcription from P_{bhuR} was absent in the Δ*rhuI* mutant under any condition tested (Fig. 4B), a result which was consistent with a model in which *rhuI* is essential for Fe-dependent P_{bhuR} activity whether or not the cell is grown in the presence or absence of hemin. In contrast, the P_{bhuR} transcription profile of the Ω cassette mutant was indistinguishable from that of wt 4169rif (Fig. 4B), thus indicating that the transcripts detected by primer extension did not originate from P_{rhuI} or other upstream promoters. Interestingly, a modest transcriptional activity was observed in RNA obtained from Fe-replete cultures of the *fur* mutation (Fig. 4B). This observation suggested that Fe-dependent repression of P_{bhuR} activity is mediated by Fur. Taken together, these results supported the hypothesis that P_{bhuR} is active under Fe-limiting conditions irrespective of heme and that P_{bhuR} requires a functional *rhuI* for transcriptional activity. These results also strongly implied that P_{bhuR} activity is repressed by Fe in a Fur-dependent manner.

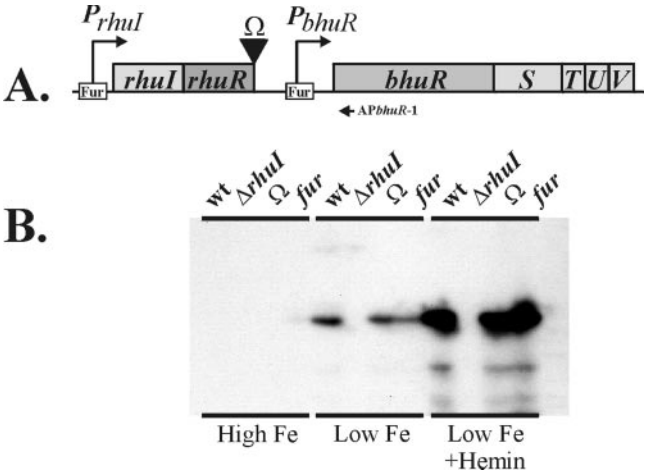

FIG. 4. Fe-dependent repression of P_{bhuR} is relieved in the *fur* mutant. (A) Schematic of *rhuIR-bhuRSTUV* locus (not drawn to scale). Arrows denote the positions of the APbhuR-1 primer used in primer extension analysis. Putative Fur boxes and the location of the Ω cassette insertion are denoted. (B) Primer extension analysis of total RNA isolated from 4169rif (wt), 4169rifΔ*rhuI* (Δ*rhuI*), 4169rifΩ (Ω), and 4169rif*fur* (*fur*). All strains were cultured in BHI broth supplemented with 36 μM $FeSO_4$ (High Fe), 100 μM EDDHA (Low Fe), or 100 μM EDDHA plus 1 μM hemin (Low Fe + hemin). The length of extended primers corresponds to extension products terminating at the A residue identified in Fig. 1B as the transcriptional start site of P_{bhuR}.

Evaluation of P_{bhuR} activity by RNase protection analysis. As a complementary approach, the transcriptional architecture of the genomic *rhu-bhu* locus was also evaluated using RNase protection. Results from primer extension analysis of 4169rif were consistent with a model in which transcription from P_{bhuR} initiates under Fe-limiting growth conditions in the absence of heme (Fig. 1B and 4B). While results from promoter-reporter analyses of 4169rifΔ*rhuI* (Fig. 2B), Pho20Ω, and Pho20Ω*fur* (Fig. 3B) were inconclusive with respect to P_{bhuR} activation, primer extension results from these mutants strongly suggested that Fe-dependent, heme-independent transcription originates from P_{bhuR}, an observation which is not consistent with previous reports of transcriptional analysis of *hurIR-bhuR* of *B. pertussis* (28). Furthermore, promoter-reporter analyses of Pho20*fur* and Pho20Ω*fur* (Fig. 3B) clearly showed that *bhuR* expression originated, at least in part, from P_{rhuI}. To explicate the details of P_{bhuR} activity in *B. avium*, the effects of Δ*rhuI* and Ω cassette mutations on transcription of *rhuIR-bhuRSTUV* were examined using RNase protection analysis. Antisense probes directed against three different regions of the *rhu-bhu* locus were designed to evaluate expression of the following different regions: (i) a region encompassing the 3′ end of *rhuI*

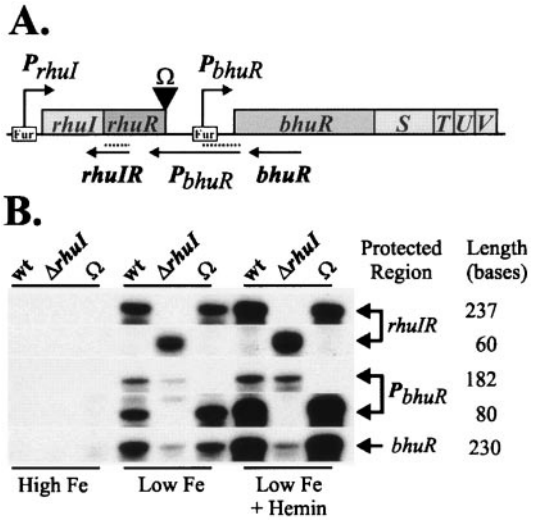

FIG. 5. Fe-dependent expression of *bhuR* from P_{bhuR} is *rhuI* dependent. (A) Schematic of *rhuIR-bhuRSTUV* locus (not drawn to scale). Arrows denote full-length protected fragments of antisense probes used in RNase protection analysis. Dotted lines represent shorter protected fragments of the indicated antisense probes. (B) RNase protection assay of total RNA isolated from 4169rif (wt), 4169rifΔ*rhuI* (Δ*rhuI*), and 4169rifΩ (Ω) cultured in BHI broth supplemented with 36 μM $FeSO_4$ (High Fe), 100 μM EDDHA (Low Fe), or 100 μM EDDHA plus 5 μM hemin (Low Fe + Hemin) using antisense probes as indicated above (A). Sizes of protected fragments in nucleotides are indicated.

and the 5′ end of *rhuR* (*rhuIR*), (ii) the entire *rhuR-bhuR* intergenic region (P_{bhuR}), and (iii) a region encompassing the 5′ end of *bhuR* (*bhuR*) (Fig. 5A).

None of the three antisense probes were protected from RNase A digestion by the addition of total RNA isolated which was obtained from Fe-replete cultures of 4169rif, indicating that transcripts from the *rhu-bhu* locus are highly repressed under these growth conditions (Fig. 5B). All three antisense probes were, however, protected by total RNA isolated from 4169rif cultured under conditions of Fe limitation (Fig. 5B). Sizes of the protected fragments in each case were determined by comparison to a commercial RNA standard ladder (data not shown). A fragment of approximately 237 nt, equivalent in size to the full-length antisense probe of the *rhuIR* region, was protected by total RNA from 4169rif, demonstrating that *rhuI* and *rhuR* are cotranscribed on a polycistronic mRNA. Likewise, total RNA from 4169rif cultured under Fe-limiting conditions protected a fragment of approximately 182 nt in length, consistent with the full-length antisense probe of P_{bhuR} which targeted the 3′ end of *rhuR*, the *rhuR-bhuR* intergenic region, and the 5′ end of *bhuR*. Protection of the entire length of this second antisense probe indicated that transcription proceeded from *rhuR* and continued through the intergenic region and into *bhuR*. The intensity of the 182-nt P_{bhuR}-protected fragment was considerably lower than the intensity of the *rhuIR*-protected fragment, suggesting that only some of the transcripts originating upstream from *rhuI* continued through the intergenic region and into *bhuR*. A 230-nt protected fragment representing the full-length antisense probe of a region within the *bhuR* ORF exhibited a signal which was higher in intensity than that of the fragment from the probe homologous to the *rhuR-bhuR* intergenic region. These results suggested that transcripts originating upstream from *rhuI* were not the sole source of *bhuR*-encoding transcripts produced by the cell. In fact, a shorter, 80-nt protected fragment was evident in experiments using the P_{bhuR} antisense probe (Fig. 5B). The length of this shorter protected fragment corresponded to the P_{bhuR} start site as determined by primer extension (Fig. 1B). Taken together, the combined intensities of the shorter 80-nt and full-length 182-nt protected fragments from the P_{bhuR} antisense probe were essentially equivalent to the signal produced by protection of the *bhuR* antisense probe. When hemin was added to the culture broth, the intensities of the shorter 80-nt P_{bhuR}-protected fragment and the *bhuR*-protected fragment increased significantly (Fig. 5B). This observation was consistent with the patterns of heme induction of *bhuR* from P_{bhuR} reported by Kirby et al. (17). A slight increase in the intensity of the *rhuIR*-protected fragment was also detected upon addition of hemin to the culture medium (Fig. 5B). These data supported the results shown in Fig. 1A that demonstrated that P_{rhuI} activity was induced by heme.

Total RNA isolated from the Δ*rhuI* mutant provided the same degree of protection of *rhuIR*-specific probes as did total RNA from 4169rif when cultured under similar growth conditions (Fig. 5B). These data confirmed that *rhuIR* expression was not autoregulated. These results also indicate that unlike P_{bhuR}, P_{rhuI} is induced by heme but not via the BhuR-RhuR-RhuI signal cascade. These data suggest the presence of an additional heme-dependent regulatory mechanism. The smaller size of the *rhuIR*-protected fragment was consistent with the size of the fragment predicted to be protected by total RNA obtained from the mutant in which the *rhuI* ORF had been removed (i.e., 4169rifΔ*rhuI*). Protection of the antisense *bhuR* probe was greatly reduced in RNA isolated from Fe-limited cultures of the Δ*rhuI* mutant cultured in the presence or absence of hemin. The pattern of protection of the antisense P_{bhuR} probe was also influenced by the Δ*rhuI* mutation. The shorter 80-nt fragment of P_{bhuR} was not protected by RNA isolated from the Δ*rhuI* mutant grown under Fe-limited conditions in the presence or absence of hemin. The larger 182-nt fragment of P_{bhuR}, however, was protected to a degree equal to the protection of the *bhuR* antisense probe (Fig. 5B). The results again confirmed that some expression of *bhuR* is dependent upon P_{rhuI} activity. The results also showed that transcription from P_{bhuR} under Fe-limiting conditions not only is *rhuI*-dependent and hemin-induced but also occurs in the absence of hemin, which is consistent with the primer extension results (Fig. 1B and 4B).

Total RNA isolated from the 4169rifΩ protected the *rhuIR* and *bhuR* antisense probes in a manner similar to that of RNA from 4169rif cells cultured under Fe-limiting conditions in either the presence or absence of hemin (Fig. 5B). Analysis of the P_{bhuR} region showed that protection of the shorter 80-nt fragment was unaffected by the Ω cassette, whereas protection of the 182-nt full-length P_{bhuR} antisense probe was eliminated (Fig. 5B). These results demonstrated that transcriptional initiation at P_{bhuR} is not dependent upon heme or P_{rhuI} and indicated that Fe-dependent, heme-independent expression of *bhuR* originates from two promoters (P_{rhuI} and P_{bhuR}). Furthermore, it is clear that P_{bhuR} activity requires *rhuI*.

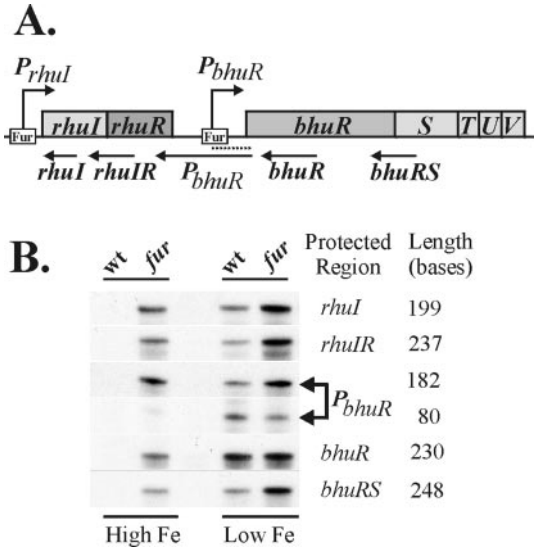

FIG. 6. Fe-dependent expression of the heme utilization locus *rhuIR-bhuRSTUV* is regulated by Fur. (A) Schematic of the *rhuIR-bhuRSTUV* locus (not drawn to scale). Arrows denote full-length protected fragments of antisense probes used in RNase protection analysis. Dotted lines represent shorter protected fragments of the indicated antisense probes. (B) RNase protection assay of total RNA isolated from 4169rif (wt) and 4169rif*fur* (*fur*) cultured in BHI broth supplemented with 36 μM $FeSO_4$ (High Fe) or 100 μM EDDHA (Low Fe) using antisense probes shown above (A). Sizes of protected fragments in nucleotides are indicated.

Fe-dependent regulation of P_{rhuI} and P_{bhuR} activities in a *B. avium fur* mutant strain. *rhuI* is Fur regulated in *B. avium* (17). Also, Fe-dependent repression of *bhuR* was found to be influenced by *fur* (17). Consistent with those observations, the *rhuR-bhuR* intergenic region was reported to contain a nucleotide sequence with weak homology to the *E. coli* Fur box consensus. This region also exhibited weak but significant titration activity in the *E. coli* Fur titration assay (FURTA) strain. Thus, experiments were designed to establish whether or not Fur had a direct role in Fe-dependent regulation of P_{bhuR} (17). Promoter-reporter assays using the Pho20*fur* and Pho20Ω*fur* strains indicated that P_{rhuI} contributed to Fur-dependent *bhuR* expression (Fig. 3). In contrast, primer extension analysis showed that both P_{rhuI} and P_{bhuR} were inactive under Fe-replete conditions and induced only when Fe was limiting (Fig. 1). Further analysis showed that P_{bhuR} in a *fur* mutant was slightly activated under Fe-replete conditions (Fig. 4B).

To further evaluate expression of the *rhu-bhu* locus in a *fur* mutant, RNase protection assays were conducted. Total RNA isolated from Fe-replete and Fe-limited cultures of 4169rif and 4169rif*fur* were utilized for these RNase protection analyses. In addition to the antisense probes used in the previous RNase protection assays (Fig. 5A), two additional probes were employed to broaden the transcriptional analysis of the *rhu-bhu* locus: an *rhuI* antisense probe which was directed against the 5′ end of *rhuI* and a *bhuRS* antisense probe which was directed against a region encompassing the 3′ end of *bhuR* and the 5′ end of *bhuS* (Fig. 6A). All five antisense probes were protected from RNase A digestion by total RNA isolated from 4169rif cultures grown under Fe-limiting conditions but were not protected by total RNA isolated from 4169rif cultured under Fe-replete conditions (Fig. 6B). These data, along with the data reported in Fig. 5B, supported a model in which the *rhu-bhu* locus including *rhuI*, *rhuR*, *bhuR*, and *bhuS* was expressed in an Fe-dependent manner. The presence of the 182- and 80-nt protected fragments of the antisense P_{bhuR} probe in reactions containing total RNA isolated from Fe-limited cultures is likely the result of readthrough transcription from P_{rhuI} and of transcription from P_{bhuR}, respectively. This pattern of expression indicated that transcriptional activities of both P_{rhuI} and P_{bhuR} were Fe-regulated events. Surprisingly, the intensity of the 182-nt protected fragment of the P_{bhuR} antisense probe was equal to the intensities of the protected fragments of the *rhuI* and *rhuIR* antisense probes (Fig. 6B). This observation was interpreted as strongly supportive of a model in which all transcripts from P_{rhuI} extended past the *rhuR-bhuR* intergenic region. This result is contrary to data reported in Fig. 5 which indicated that not all transcripts from P_{rhuI} proceeded through the intergenic region into *bhuR*. Although the discrepancy between the two experiments has not been resolved, it is possible that the difference is indicative of an unknown mechanism controlling differential transcriptional termination in the *rhu-bhu* locus for which the regulatory conditions are yet to be determined.

Total RNA isolated from Fe-replete cultures of 4169rif*fur* protected the antisense probes directed against regions of the *rhu-bhu* locus. Also, Fe-dependent repression from *rhuI* to *bhuS* was relieved in the *fur* mutant. All fragments of antisense probes protected by RNA from Fe-replete cultures of the *fur* mutant are equally protected by RNA from 4169rif cells grown under Fe-limited conditions, with one exception. The 80-nt protected fragment, which likely is representative of transcription from P_{bhuR}, was only slightly evident in the *fur* mutant when cells were cultured under Fe-replete conditions. Longer exposure of the autoradiograph revealed that this fragment was protected by RNA from Fe-replete cultures of 4169rif*fur* but not by RNA from Fe-replete cultures of 4169rif (data not shown). Additionally, β-galactosidase activity from the P_{bhuR}::*lacZYA* reporter in pDJM41 was measured in 4169rif and 4169rif*fur* strains, both cultured under Fe-replete and Fe-limited growth conditions (Fig. 7B). The results show that Fe-dependent repression of P_{bhuR} is relieved in the absence of *fur*. This evidence, taken together with previous results reported by Kirby et al. (17), strongly implicated *fur* in regulating Fe-dependent P_{bhuR} activity.

DISCUSSION

In *B. avium*, heme-dependent expression of *bhuRSTUV* requires the outer membrane heme receptor BhuR, the ECF σ factor RhuI, and the ECF σ factor activator RhuR (16, 17, 22). Paradoxically, this regulatory mechanism requires *bhuR* to be expressed in the absence of heme inducer. In *B. pertussis* and *B. bronchiseptica*, heme-independent expression of *bhuR* was found to originate from P_{hurI}, an Fe-regulated promoter located immediately upstream from the *hurIR-bhuRSTUV* gene cluster (28). This gene cluster is orthologous to the *B. avium* heme utilization locus *rhuIR-bhuRSTUV* (22). Here, we present evidence that in *B. avium*, heme-independent tran-

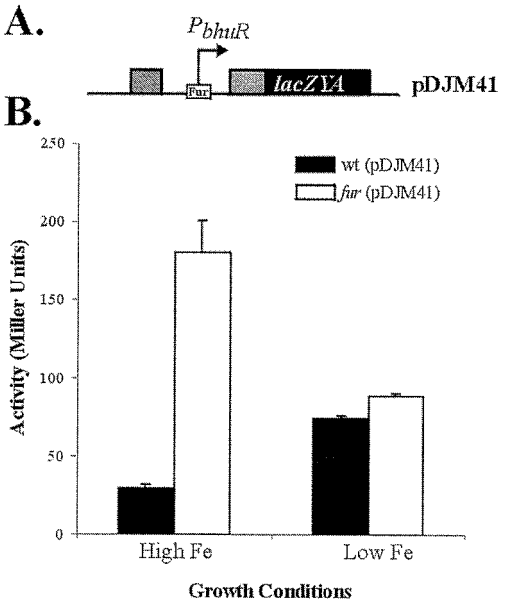

FIG. 7. Fur-dependent repression of P_{bhuR}. (A) pDJM41 encodes a 199-bp segment of DNA encompassing the *rhuR-bhuR* intergenic region containing P_{bhuR} (thick line) which is fused to *lacZYA* (shaded box) (not drawn to scale). (B) Both wt 4169rif and 4169rif*fur* were transformed with pDJM41. Strains were cultured in BHI broth supplemented with 36 μM $FeSO_4$ (High Fe) or 100 μM EDDHA (Low Fe). β-Galactosidase activities were measured from log-phase cultures.

scription of *bhuR* originates from P_{bhuR}, a proximal RhuI-dependent promoter, as well as from P_{rhuI}, a distal RhuI-independent promoter. This mechanism ensures the synthesis of adequate amounts of BhuR in the absence of heme induction for subsequent heme sensing. Data also point toward the likelihood that both promoters are repressed by Fur under Fe-sufficient conditions and suggest the necessity for swift repression of the heme acquisition system once the demand for Fe has been met.

Transcription from P_{rhuI} initiates 28 bases upstream from the *rhuI* ORF and is evident only when Fe is limiting (Fig. 1A). Transcription from P_{rhuI} is unaffected by a Δ*rhuI* mutation, indicating that this promoter is not autoregulated and, therefore, not likely to be activated by heme via the BhuR-RhuR-RhuI signal cascade (Fig. 5B). The observed effect that the addition of hemin to the medium slightly enhanced P_{rhuI} activity was unexpected and suggests the presence of an additional heme-dependent signal cascade. An alternative explanation, however, in which P_{rhuI} activity is indirectly affected by heme cannot be ruled out. Further examination of P_{rhuI} is needed to assess the role of heme in *rhuI*-independent expression from this promoter. The P_{rhuI} transcriptional start site is optimally spaced from σ^{70}-like promoter elements and is located within a putative Fur box in a region that exhibited positive FURTA activity (17). Alkaline phosphatase activities of the Pho20, Pho20*fur*, and Pho20Ω*fur* strains support Fur-dependent regulation of P_{rhuI} and indicated that at least a portion of the transcripts from P_{rhuI} read through the *rhuR-bhuR* intergenic region (Fig. 3B). RNase protection analysis provides evidence that the readthrough transcripts include the coding regions of *bhuR* and *bhuS*, at the very least, and likely include the other genes of the *bhuR* locus (Fig. 5B and 6B). These results, consistent with those reported for *B. pertussis* and *B. bronchiseptica* (28), describe a suitable mechanism for the heme-independent expression of *bhuR* observed in all three species.

Transcriptional analyses of the *rhuR-bhuR* intergenic region indicated that P_{rhuI} is not the sole active promoter driving *bhuR* expression. Transcription from P_{bhuR} starts 37 bases upstream from *bhuR* (Fig. 1B). RNase protection analysis using an antisense probe directed against the *rhuR-bhuR* intergenic region yielded, in addition to a 182-nt protected fragment that represents readthrough transcription, a shorter 80-nt protected fragment whose length is coincident with the P_{bhuR} transcriptional start site (Fig. 5B). Surprisingly, transcription from P_{bhuR} occurs under Fe-limiting conditions in the absence of heme, although to a much lesser degree than when heme is present (Fig. 2B, 4B, and 5B). Previous studies have shown that heme and heme-containing proteins are inducers of *bhuR* expression (22). Mutational studies confirmed that RhuI is required for P_{bhuR} activity and showed that RhuI can also function in the absence of heme inducer (16, 17) (Fig. 3B, 4B, and 5B). Previous experiments have shown that, when overexpressed, RhuI constitutively activates *bhuR* expression (17). It is possible that RhuI has a basal activity for P_{bhuR} induction that is enhanced in the presence of heme through signaling via BhuR and RhuR.

In *B. avium*, both P_{rhuI} and P_{bhuR} participate in heme-independent *bhuR* expression. A similar *rhuI*-dependent, heme-independent P_{bhuR} activity, observed in primer extension analysis of *B. bronchiseptica*, was reported in a previous study, but no explanations for this activity were proffered (28). Instead, heme-independent expression of *bhuR* in *B. bronchiseptica* and *B. pertussis* was reported to originate exclusively from P_{hurI} (28). It is unexpected that transcription from P_{bhuR} in *B. avium* should be controlled differently from that in *B. pertussis* and *B. bronchiseptica*. In all three organisms, expression of *bhuRSTUV* is induced by heme via a system that requires BhuR, the ECF σ factor (HurI), and the coregulator (HurR) (RhuI and RhuR for *B. avium*), the expression of which are Fe regulated in a Fur-dependent manner. The differences in the regulation of *bhuR* in these organisms could be attributable to a region of DNA found in the *hurIR-bhuR* intergenic region of *B. pertussis* and *B. bronchiseptica* but absent in the *rhuR-bhuR* intergenic region of *B. avium*. This span of additional DNA could contain structural *cis*-acting elements that proffer additional regulation.

Employing two promoters to control heme-independent *bhuR* expression would appear redundant for *B. avium*. It is feasible, however, that the regulatory system involving the two promoters evolved to enable the bacterium to respond to different sources of heme. Several observations support this model: (i) acquisition of both heme and heme-containing proteins (e.g., hemoglobin, myoglobin, catalase, etc.) requires *bhuR* (22); (ii) growth of *B. avium* with hemin as its sole Fe source does not require *rhuR*, but a Δ*rhuR* mutant has reduced capacity to utilize hemoglobin (16); and (iii) BhuR is expressed in two forms in the outer membrane, a 91-kDa protein encoded by the entire *bhuR* ORF and a truncated 82-kDa protein in which the N-terminal 104 amino acids of the full-size protein have been removed by a proteolytic cleavage (22). The 82-kDa

form is preferentially expressed when *B. avium* encounters heme. The interplay between P_{rhuI} and P_{bhuR} may be necessary to facilitate a response by *B. avium* to different biological sources of heme. Experiments to evaluate this model are ongoing.

Transcriptional analysis using RNA from Fe-replete cultures of strain 4169rif*fur* revealed that Fe-dependent repression of the *rhuIR-bhuRSTUV* locus was relieved in a *fur* mutant (Fig. 6B). Protected fragments representing transcripts from *rhuI* and *rhuR* from 4169rif*fur* cultured under Fe-replete conditions were equally abundant, thus confirming that *rhuI* and *rhuR* are transcribed on a single message that is repressed by *fur* in an Fe-dependent manner. It is clear from the RNase protection experiments that synthesis of this polycistronic mRNA sometimes extends into *bhuR* and *bhuS*. Although not determined in these experiments, it is likely that synthesis of the polycistronic mRNA extends into *bhuTUV*, thus encoding all proteins of the heme uptake system. However, RNase protection experiments also demonstrated that transcripts originating from P_{rhuI} sometimes terminate after *rhuI* (Fig. 5B). The conditions and the cognate signal(s) that regulate whether polymerization of transcripts continues or terminates after synthesis of *rhuI* and *rhuR* have not been established. There is no evidence of a *bhuR*-associated feedback regulation. However, it is possible that one of the genes located downstream of *bhuR* may participate in regulating synthesis of the polycistronic mRNA. The role of downstream genes (*bhuSTUV*) in the control of readthrough transcription, however, is yet to be investigated.

It is clear that Fur has a potent role in governing expression of *rhuI*, *rhuR*, *bhuR*, and *bhuS*. A region with weak (8 of 19 nucleotides) homology to the consensus Fur box was located between the putative −35 and −10 elements of P_{bhuR} (17). The Fur box appears to be functionally active, since a segment of DNA containing this region exhibited weak FURTA activity (17), implying that it has the capacity to functionally interact with Fur. Transcriptional analyses of RNA isolated from Fe-replete cultures of a *fur* mutant of *B. avium* supported the hypothesis that Fur represses the RhuI-dependent P_{bhuR} (Fig. 4B). Furthermore, β-galactosidase expressed from P_{bhuR} on pDJM41, which does not harbor DNA containing P_{rhuI}, is still Fe responsive, even in the Δ*rhuI* mutant (Fig. 3B). Although not unequivocal, these data provided strong evidence that P_{bhuR} activity is highly dependent upon Fe, likely via an interaction with Fur. It was predicted that experiments utilizing the Ω cassette would be useful in isolating P_{bhuR} from P_{rhuI} for evaluating the effect of Fur on P_{bhuR} activity. Unfortunately, any effects that Fur exerted on P_{bhuR} were masked in these experiments by an apparent artifactual Fe-independent transcriptional activity originating from within the Ω cassette. For that reason, the Ω cassette is not a particularly desirable tool for isolating transcriptional units in the bordetellae. As a better alternative strategy for investigating putative interactions of Fur with P_{bhuR}, future experiments will evaluate P_{bhuR} activity in an engineered mutant in which P_{rhuI} is absent.

The need for Fur-dependent regulation at P_{rhuI} and P_{bhuR} sites may indicate that rapid downregulation of expression of *bhuRSTUV* is desirable when heme and the other components of the signal transduction cascade are still present in the cell but when the Fe requirements of the cell have already been met. Fur may be a strong participant in a regulatory scheme which determines whether transcription from P_{rhuI} terminates after *rhuR* or reads through to *bhuRSTUV*. Given the multiple forms of BhuR, the multiple inducers that stimulate *bhuR* expression, the multiple promoters that drive this expression, and the complexity of *bhuR* regulation, it is apparent that a complete description of the regulation and functioning of the *rhuIR-bhuRSTUV* heme utilization locus is yet to be fully revealed. Elucidation of the details of the RhuI-RhuR-BhuR signaling cascade and the characterization of the genes downstream from *bhuR* may provide valuable insights into Fur regulation, Fe acquisition, and virulence of the bordetellae.

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