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Integration of Environmental Signals Controls Expression of *Bordetella* Heme Utilization Genes

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The *Bordetella pertussis* heme utilization gene cluster *hurIR bhuRSTUV* encodes regulatory and transport functions required for assimilation of iron from heme and hemoproteins. Expression of the *bhu* genes is iron regulated and heme inducible. The putative extracytoplasmic function (ECF) σ factor, HurI, is required for heme-responsive *bhu* gene expression. In this study, transcriptional activation of *B. pertussis bhu* genes in response to heme compounds was shown to be dose dependent and specific for heme; protoporphyrin IX and other heme structural analogs did not activate *bhu* gene expression. Two promoters controlling expression of the heme utilization genes were mapped by primer extension analysis. The *hurI* promoter showed similarity to σ^{70}-like promoters, and its transcriptional activity was iron regulated and heme independent. A second promoter identified upstream of *bhuR* exhibited little similarity to previously characterized ECF σ factor-dependent promoters. Expression of *bhuR* was iron regulated, heme responsive, and *hurI* dependent in *B. pertussis*, as shown in a previous study with *Bordetella bronchiseptica*. Further analyses showed that transcription originating at a distal upstream site and reading through the *hurR-bhuR* intergenic region contributes to *bhuR* expression under iron starvation conditions in the absence of heme inducer. The pattern of regulation of the readthrough transcript was consistent with transcription from the *hurI* promoter. The positions and regulation of the two promoters within the *hur-bhu* gene cluster influence the production of heme transport machinery so that maximal expression of the *bhu* genes occurs under iron starvation conditions only in the presence of heme iron sources.

The innate immune system of the human host defends against invading microorganisms in part by sequestering iron, a nutrient essential for virtually all living cells. The majority of host iron is maintained intracellularly in the form of hemoproteins, while extracellular iron is bound by the host glycoproteins transferrin and lactoferrin (44, 49). Successful microbial pathogens have evolved mechanisms to overcome host iron restriction (21, 32, 46), including production and utilization of low-molecular-weight iron chelators termed siderophores (40), utilization of siderophores produced by other organisms, and direct removal of iron from host proteins via specific bacterial cell surface receptors (11, 59).

In gram-negative and some gram-positive bacterial species, genes encoding iron transport systems are repressed when intracellular iron levels are high by the Fur protein with ferrous iron as the corepressor (17, 22). When bacterial cells encounter an iron-limiting environment such as the human host, their intracellular iron stores are depleted, resulting in derepression of iron acquisition genes. Fur derepression is sufficient for full expression of the genes in certain iron uptake systems, while in other systems, positive transcriptional regulation requiring the presence of the cognate iron source is also necessary for maximal gene expression (12). Positive regulators of iron acquisition systems are of three main classes: AraC-like proteins (4, 9, 18, 24, 43), two-component signal transduction systems (14, 50), and extracytoplasmic function (ECF) σ factors (1, 13, 30, 31, 57).

ECF σ factors are members of the σ^{70} superfamily of bacterial sigma factors and are utilized by diverse species to regulate genes in response to extracytoplasmic stimuli (37, 45). ECF σ factors involved in regulating iron stress responses have been termed members of the iron starvation subfamily of ECF regulators (58). These ECF σ factors and their specific anti-σ factors are produced under iron-limiting conditions, but the σ factors remain inactive until the cognate iron source is sensed in the environment. In the presence of the appropriate iron source, a signaling cascade is initiated at the cell surface by the cognate outer membrane receptor. The signal is transduced to the anti-σ factor, which then either releases or activates the σ factor, allowing it to associate with core RNA polymerase and initiate transcription of genes encoding iron acquisition functions (6, 58). Members of the iron starvation family of ECF σ factors include FecI (1), PupI (31), and PvdS (13), which regulate a subset of iron uptake genes in *Escherichia coli*, *Pseudomonas putida*, and *Pseudomonas aeruginosa*, respectively. Recently, the putative ECF σ factors HurI of *Bordetella pertussis* and *Bordetella bronchiseptica* (57) and RhuI of *Bordetella avium* (30) were shown to regulate expression of heme iron transport genes.

Since greater than 90% of the iron within the human body is associated with heme and hemoproteins (42), bacteria that can access these compounds in vivo and utilize host heme iron have a significant nutritional advantage. *Vibrio cholerae* (25, 26), pathogenic *E. coli* (55), *Shigella* species (36), *Yersinia* species (53, 54), and *P. aeruginosa* (41) produce TonB-dependent cell surface receptors and ATP-binding cassette transporters that allow utilization of heme, hemoglobin, and other hemopro-

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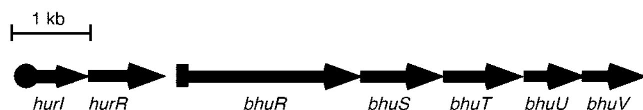

FIG. 1. *B. pertussis* and *B. bronchiseptica* heme iron utilization genetic locus. The *hurI* and *hurR* genes encode a σ factor and cytoplasmic membrane protein (anti-σ factor), respectively. The *bhuRSTUV* genes encode components of the heme iron transport machinery. BhuR is the outer membrane receptor, and BhuS is a predicted heme binding protein; BhuT, BhuU, and BhuV are components of the periplasmic binding protein-dependent ATP-binding cassette transporter system. The solid circle upstream of *hurI* represents a predicted σ^{70}-like promoter, while the solid rectangle upstream of *bhuR* denotes a putative HurI-dependent, heme-responsive promoter. The arrows indicate the direction of transcription.

teins. A second type of heme uptake system, employed by species such as *Serratia marcescens* (33), *Yersinia pestis* (47), and *P. aeruginosa* (41), involves production and secretion of small heme-binding proteins termed hemophores that obtain and ferry host heme to specific bacterial cell surface receptors.

B. pertussis, the causative agent of the human disease whooping cough, and *B. bronchiseptica*, a closely related mammalian respiratory pathogen, possess multiple systems for iron retrieval under iron-limiting environmental conditions. They produce the siderophore alcaligin (8, 20, 28, 38) and are capable of using siderophores produced by other organisms (3). Both species possess the heme utilization gene cluster *bhuRSTUV*, which encodes transport functions required for assimilation of iron from heme and hemoproteins (Fig. 1) (56). *B. avium*, a more distantly related pathogen of turkeys and chickens, has an orthologous gene cluster encoding a functional heme utilization system (39). Expression of *B. pertussis* and *B. bronchiseptica bhu* genes is regulated by iron and the presence of heme via Fur and the ECF regulators encoded by the *hurIR* genes, located immediately upstream of the *bhu* gene cluster (56, 57).

We showed in a previous study that HurI, a putative ECF σ factor, is required for heme-activated *bhuR* transcription and for maximal levels of heme utilization (57). In the present study, the kinetics of the transcriptional response to heme inducer and the structural characteristics of the inducer were examined. We have identified transcriptional start sites for the iron-regulated *hurI* and heme-inducible *bhuR* genes and have demonstrated the *hurI* dependence of heme-responsive *bhuR* expression in *B. pertussis*. Furthermore, iron-regulated *bhuR* transcription in the absence of heme was assessed, and it was found that transcription from an upstream promoter, reading through the *hurR-bhuR* intergenic region, contributes to *bhuR* expression. These data support a model for transcriptional regulation of heme utilization genes that allows *Bordetella* cells to sense heme and respond by maximally producing the heme transport machinery.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Bordetella* strains and recombinant plasmids used in this study are listed in Table 1. *E. coli* DH5α (Invitrogen, Gaithersburg, Md.) was used as the host strain in routine cloning procedures. Plasmid vectors pGEM3Z (Promega, Madison, Wis.) and pRK415 (29) were used in the construction of recombinant plasmids. A pRK415 derivative, plasmid pRK40 (57), carries a promoterless *trp′-′lacZ* gene and was used to construct all *bhuR-lacZ* transcriptional fusions (Table 1).

Growth media and chemical solutions. Luria-Bertani (LB) (48) broth or agar plates were used to culture *E. coli* strains. *B. pertussis* strains were cultured on Bordet-Gengou (BG) agar (5); *B. bronchiseptica* strains were cultured on LB agar. All *Bordetella* liquid cultures were grown in Stainer-Scholte (SS) minimal medium (51, 52). SS medium was deferrated by Chelex100 (Bio-Rad, Richmond, Calif.) as described previously (2). Iron-depleted SS medium contained no iron supplements, while iron-replete SS medium was supplemented with $FeSO_4$ to a final concentration of 36 μM. Bovine hemin chloride (Sigma, St. Louis, Mo.) was maintained as a 1 mM stock solution as described previously (56) and added to iron-depleted cultures at a final concentration of 5 μM unless otherwise indicated. Ethanolic stock solutions of chlorophyll *a* (Sigma) were prepared at a concentration of 1 mM; aqueous solutions of protoporphyrin IX (PPIX) and cytochrome *c* (both from Sigma) were maintained at concentrations of 400 μM and 500 μM, respectively, and zinc-PPIX (Sigma) was dissolved in *N,N*-dimethyl formamide at a concentration of 800 μM. Each porphyrin compound was added to liquid cultures at a final concentration of 5 μM unless otherwise indicated. Tetracycline and ampicillin were used at final concentrations of 15 μg/ml and 100 μg/ml, respectively.

Bacterial culture conditions. *B. pertussis* and *B. bronchiseptica* strains were grown on agar plates and subcultured to iron-replete SS medium. *B. bronchiseptica* cells were grown with shaking at 37°C for 24 h, washed, and inoculated at a dilution of 1:200 to iron-replete and iron-depleted SS medium. After 18 h of growth, hemin was added as appropriate to iron-depleted cultures. All cultures were harvested for β-galactosidase assays or RNA isolation 4 h after the addition of hemin (after a total of 22 h of growth). A similar procedure was used to culture *B. pertussis* strains except that iron-replete SS cultures were grown for 36 h;

TABLE 1. *Bordetella* strains and reporter plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
UT25Sm1	*B. pertussis*; spontaneous streptomycin-resistant derivative of wild-type strain UT25	19
PM8	*B. pertussis* UT25Sm1 Δ*hurI*	57
B013N	*B. bronchiseptica*; spontaneous nalidixic acid-resistant derivative of wild-type B013	2
Plasmids		
pRK40	pRK415 with 3.3-kb *Eco*RI-*Hin*dIII *trp′-′lacZ* insert fragment; Tet^r	57
pRK41	pRK40 with 0.5-kb *B. pertussis* UT25 *′hurR-bhuR′* insert fragment; *bhuR-lacZ* transcriptional fusion; Tet^r	57
pRK42	pRK40 with 2.1-kb *B. pertussis* UT25 *hurIR-bhuR′* insert fragment; *hurIR bhuR-lacZ* transcriptional fusion; Tet^r	57
pRK45	pRK40 with 0.3-kb *B. pertussis* UT25 *bhuR′* insert fragment; *bhuR-lacZ* transcriptional fusion; Tet^r	This study
pRK47	pRK42 with 12-nt block substitution in *bhuR* promoter region; *hurIR bhuR-lacZ* transcriptional fusion; Tet^r	This study
pRK48	pRK42 with unique *Bgl*II site in *hurR-bhuR* intergenic region; *hurIR bhuR-lacZ* transcriptional fusion; Tet^r	This study
pRK49	pRK48 with Ω Cm cassette inserted into *Bgl*II site, *hurIR* Ω Cm *bhuR-lacZ* transcriptional fusion; Tet^r	This study
pRK50	pRK40 with *B. pertussis* UT25 0.38-kb *bhuR′* insert fragment; *bhuR-lacZ* transcriptional fusion; Tet^r	This study
pRK51	pRK40 with *B. pertussis* UT25 0.44-kb *′hurR-bhuR′* insert fragment; *bhuR-lacZ* transcriptional fusion; Tet^r	This study

subcultures were inoculated at an initial optical density (600 nm) of 0.08 and grown for 24 h prior to hemin addition.

RNA isolation and primer extension analysis. Total RNA was harvested from cultures by a modification (27) of the acid-guanidinium thiocyanate-phenol-chloroform extraction method of Chomczynski and Sacchi (10). Primer extension reactions contained 25 μg of RNA, 1 pmol of ^{32}P-end-labeled primer, 1X Superscript II buffer (Stratagene, La Jolla, Calif.), 1 mM deoxynucleoside triphosphate mixture, 10 μM dithiothreitol, 1 mg of bovine serum albumin per ml, in a total reaction volume of 20 μl. This mixture was heated to 70°C for 5 min to denature the RNA, hybridized at 45°C for 30 min, and cooled to 37°C for 10 min. Superscript II RNase H^- reverse transcriptase (10 units) (Stratagene) was added to each reaction, which was incubated for an additional 30 min at 37°C. The primer extension reaction was stopped, and primer-extended cDNA was isolated by standard methods (48). Prior to loading on an 8% polyacrylamide gel, the products were denatured by boiling for 5 min. One-half of the final volume was loaded on the gel next to a nucleotide sequencing ladder generated by appropriate primers with plasmid DNA templates.

Reverse transcription-PCR analysis. Reverse transcription reactions with *Bordetella* RNA as templates were performed as described for primer extension, except that nonradiolabeled primer was used. After reverse transcription, the mixture was diluted by addition of an equal volume of distilled water, and 2 μl was used as the template for PCR. The following components were used in the PCR: water to a total volume of 50 μl, 1X Pfu Turbo buffer (Stratagene), 800 μM deoxynucleoside triphosphate mixture, 2 μl of diluted reverse transcription reaction, 8 pmol of each primer, 5% dimethyl sulfoxide, 1 unit of Pfu Turbo DNA polymerase (Stratagene). The thermal cycler was programmed for one cycle of denaturation at 96°C for 5 min, 30 cycles of denaturation at 96°C for 1 min, primer annealing at 62°C for 1 min, and extension at 72°C for 30 s, and one cycle at 72°C for 10 min.

Genetic methods. *Bordetella pertussis* nucleotide sequence data were produced by the *Bordetella* Sequencing Group at the Sanger Centre (http://www.sanger.ac.uk/Projects/B_pertussis/). Other nucleotide sequences were obtained from GenBank at the National Center for Biotechnology Information at the National Library of Medicine.

Reporter plasmid pRK40 and *bhuR-lacZ* plasmids pRK41 and pRK42 were described previously (57). β-Galactosidase assays of cells carrying reporter plasmids were performed by a modification (7) of the method of Miller (35). The results reported are representative of at least two experimental trials. Deletion derivatives of the *bhuR* promoter fragment were generated by PCR with *B. pertussis* cosmid pCPbhu1 (carrying *hurIR bhuRSTUV*) (56) as the template. The source of the Ω chloramphenicol (Cm) cassette used to construct the terminator insertion in plasmid pRK49 was mini-Tn*5* Cm (15).

The block substitution and *Bgl*II site insertion in plasmids pRK47 and pRK48, respectively, were constructed by whole-plasmid PCR mutagenesis by a method described previously (60). Briefly, primers that were antisense to one another were designed to be complementary to the *hurR-bhuR* intergenic region except for the bases to be substituted. Primers mECF1 and mECF2 contained a 12-nucleotide block substitution in the center of each primer, with 16 nucleotides of complementarity to the template DNA flanking both sides of the mutation. Primers Bgl1 and Bgl2 contained three single-nucleotide substitutions to create a *Bgl*II restriction site. These primer sequences were as follows: mECF1, 5′-CGTGCCTGCTCTCGATCCCTTTCCTTCTTCATGGTTTACGCTTGC-3′; mECF2, 5′-AAGCGTAAACCATGAAGAAGGAAAGGGATCGAGAGCAGGCACGAG-3′; Bgl1, 5′-CGGCAAAAAAAATTCCAGATCTCTGTCCGGTTTCGACG-3′; and Bgl2, 5′-CGTCGAAACCGGACAGAGATCTGGAATTTTTTTTGCCG-3′.

For whole-plasmid PCR mutagenesis, the following components were mixed in order: water to a total volume of 50 μl, 100 ng of p3Z102 plasmid DNA, 50 pmol of each primer, 1 mM deoxynucleoside triphosphate mixture, 1X Pfu Turbo buffer (Stratagene), 5% dimethyl sulfoxide, and 2.5 U of Pfu Turbo DNA polymerase (Stratagene). The thermal cycler was programmed for one cycle of denaturation at 96°C for 5 min, 16 cycles of denaturation at 96°C for 1 min, primer annealing at 55°C for 1 min, and extension at 68°C for 10 min. Following the PCR, *Dpn*I was added to digest the methylated parental template DNA. *E. coli* DH5α was transformed with 10 μl of the reaction, and plasmids from several independent transformants were sequenced to identify plasmids containing the desired mutations.

RESULTS

Temporal analysis of *bhuR* induction. The transcriptional response of iron-starved *Bordetella* cells to various heme concentrations was monitored to determine the sensitivity and time course of *bhu* gene activation. Wild-type *B. bronchiseptica* B013N cells carrying the *hurIR bhuR-lacZ* plasmid pRK42 were cultured in parallel in iron-replete SS medium and iron-depleted SS medium with or without hemin. Cells grown in iron-replete medium showed low levels of β-galactosidase activity (≈400 Miller units) that remained constant for the duration of the experiment (data not shown). Cells grown in iron-depleted medium without hemin showed ≈2-fold-higher levels of reporter gene activity compared with iron-replete cells, demonstrating iron-regulated *bhu* gene expression.

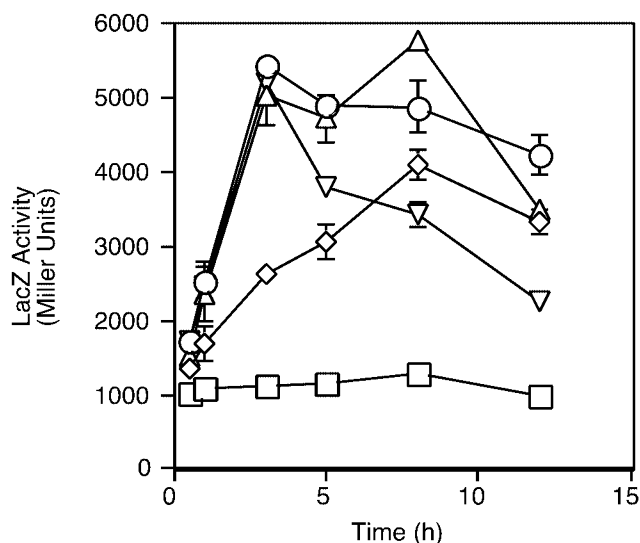

FIG. 2. Analysis of induction kinetics of *bhuR* heme-responsive transcription. *B. bronchiseptica* cells carrying *hurIR bhuR-lacZ* reporter fusion plasmid pRK42 were cultured in iron-depleted SS medium with or without (squares) hemin. Parallel cultures were assayed for β-galactosidase activity at the indicated times after addition of hemin to the following concentrations: 20 μM (inverted triangles); 5 μM (triangles); 1.25 μM (circles); and 0.32 μM (diamonds).

Replicate cultures of iron-starved cells were exposed to concentrations of hemin ranging from 0.32 μM to 20 μM (Fig. 2), and transcription of *bhuR* was activated in response to all concentrations of hemin tested. Interestingly, the lowest concentration of hemin (0.32 μM) did not measurably stimulate the growth of iron-starved cells (data not shown) but did induce *bhuR* transcription (Fig. 2), indicating that heme responsiveness and *bhuR* activation are highly sensitive. The induction kinetics of *bhuR* transcriptional activation varied with the concentration of hemin provided. Cells exposed to a low concentration of hemin (0.32 μM) showed a modest induction that slowly increased to a maximum at ≈8 h after hemin addition. Cells induced by intermediate heme concentrations (1.25 or 5 μM) showed higher peak levels of transcription that increased rapidly within 2 h and declined from maximum levels after 8 h of heme exposure. Cells given the highest dose of heme (20 μM) showed a pattern of rapid but transient induction followed by a sharper decrease in transcriptional activity, consistent with uptake of heme iron resulting in Fur-mediated repression of the fusion gene. The cultures exhibiting the highest sustained levels of *bhuR* transcription were those exposed to intermediate concentrations of hemin.

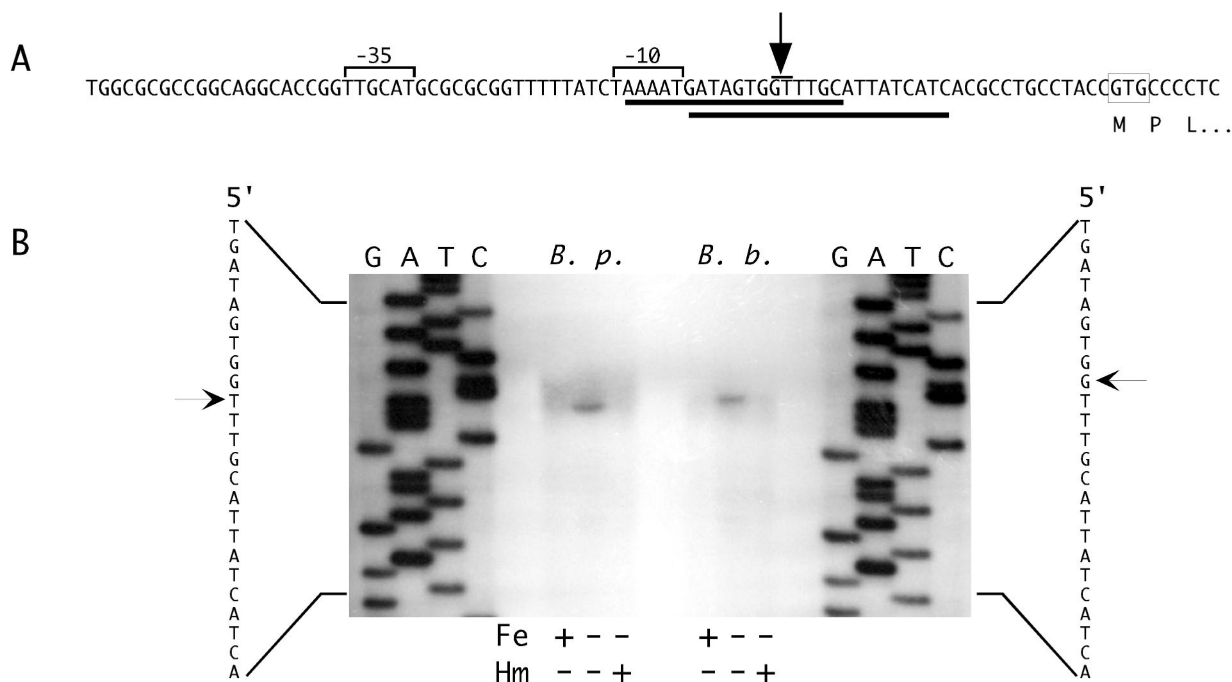

FIG. 3. Mapping of the *hurI* promoter by primer extension. (A) The nucleotide sequence and features of the *B. pertussis hurI* promoter region are shown. The predicted *hurI* GTG start codon is boxed. Solid lines below the sequence indicate predicted Fur binding sites. The arrow points to a bar above the +1 positions for the *B. pertussis* and *B. bronchiseptica hurI* genes. The −10 and −35 promoter elements are designated by brackets. (B) The autoradiogram shows the results of primer extension analysis of total RNA isolated from *B. pertussis* (*B. p.*) and *B. bronchiseptica* (*B. b.*) cells cultured under iron-replete (Fe+, Hm−), iron-depleted (Fe−, Hm−), and iron-depleted with hemin supplementation (Fe−, Hm+) conditions. Arrows designate the *hurI* transcriptional initiation sites in *B. pertussis* (left) and *B. bronchiseptica* (right).

Analysis of inducer specificity. Initiation of the signaling cascade that results in *bhu* gene transcriptional activation involves recognition of heme by the BhuR receptor protein (57). As a means to elucidate the structural requirements for BhuR inducer recognition, molecules structurally similar to heme were tested for their ability to induce *bhuR* transcription. Cytochrome *c* is a hemoprotein in which the heme moiety is covalently linked to the cytochrome protein. Although intact cytochrome *c* cannot supply nutritional iron to *Bordetella* cells (data not shown), it was hypothesized that recognition of heme at the cell surface, independent of transport, could lead to signaling and transcriptional activation of *bhuR*. PPIX, the heme biosynthetic precursor lacking a coordinated iron atom, zinc-PPIX, and chlorophyll *a*, a porphyrin with a coordinated magnesium atom, all bear significant structural similarity to heme.

To test *bhuR* transcriptional responsiveness to these heme analogs, *B. bronchiseptica* B013N(pRK42) was grown in iron-depleted SS medium and exposed to chlorophyll *a*, PPIX, zinc-PPIX, cytochrome *c*, or hemin. In multiple experiments, cells exposed to hemin showed at least a fourfold induction of *bhuR* transcription over levels exhibited by iron-starved cells. The highest level of induction in response to any other compound tested was a 1.5-fold induction in response to PPIX (data not shown). To further assess whether PPIX was a weak inducer of *bhuR* transcription, iron-starved B013N(pRK42) cells were exposed to PPIX concentrations of 5, 10, 25, and 50 μM and *bhuR* transcriptional activity was monitored. In contrast to the response to hemin (Fig. 2), there was no dose-dependent transcriptional activation in response to PPIX (data not shown), indicating that BhuR recognition of inducer is highly specific for the porphyrin ring with bound iron.

Mapping of the transcriptional initiation site for *hurI*. To elucidate the genetic mechanisms mediating inducible expression of heme utilization genes, the positions and features of promoters within the heme utilization gene cluster were defined. The *hurI* and *hurR* genes encode a putative ECF σ factor and cytoplasmic membrane regulator, respectively. In previous studies (56, 57), potential σ^{70}-like promoter elements and Fur binding sites were identified upstream of *hurI* (shown in Fig. 3A), and functional Fur binding activity in this region was demonstrated, suggesting that *hurI* transcription was iron repressible.

To directly examine *hurI* expression and identify the *hurI* transcription initiation site, total RNA isolated from wild-type *B. pertussis* UT25Sm1 and wild-type *B. bronchiseptica* B013N cells was analyzed in primer extension experiments. A *hurI* transcript was undetectable in cells grown under iron-replete conditions but was present in RNA isolated from iron-starved cells (Fig. 3B), demonstrating iron regulation at the *hurI* promoter. Addition of heme to iron-starved cultures resulted in a significant reduction in *hurI* transcript levels, suggesting that the iron requirements of the cells were satisfied by the added heme and that Fur repression of *hurI* was resumed.

A single major *hurI* transcription initiation site was observed in both *B. pertussis* and *B. bronchiseptica*. In *B. pertussis*, the site corresponded to a T residue that was 27 nucleotides upstream of the predicted *hurI* start codon (Fig. 3A), while in *B. bron-*

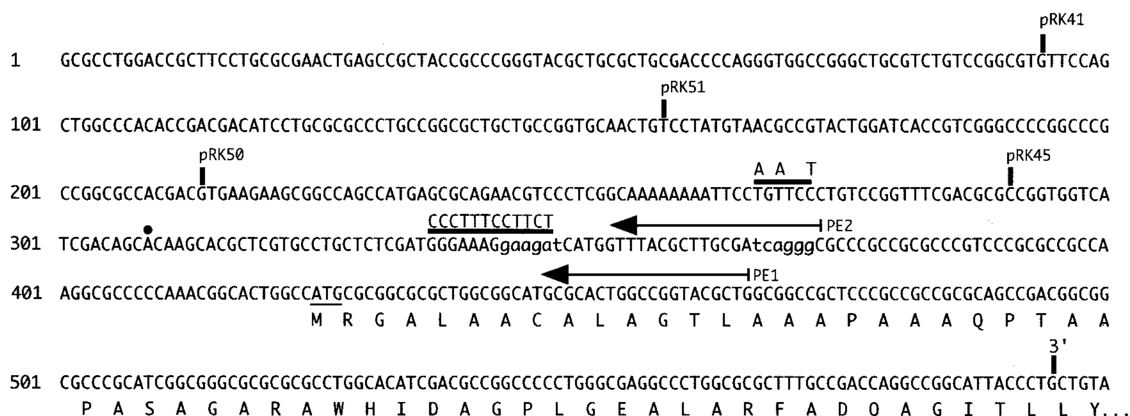

FIG. 4. Features of the *bhuR* promoter region. The nucleotide sequence of the *bhuR* upstream region and 5′ *bhuR* coding sequences (GenBank accession number AY032627) are shown. Solid vertical lines labeled pRK41, pRK51, pRK50, and pRK45 denote the 5′ limits of the *bhuR* promoter region used to construct the corresponding *bhuR-lacZ* plasmid-borne fusions (Table 1). The vertical line labeled 3′ indicates the *lacZ* fusion junction for all *bhuR-lacZ* constructs. Nucleotides 343 to 348 and 367 to 372 shown in lowercase letters represent ECF σ-like −35 and −10 elements that were predicted based on similarity to other promoters. The horizontal bar over nucleotides 336 to 347 shows the position of the block substitution mutation constructed in plasmid pRK47; the bar over nucleotides 266 to 271 shows the position of the *Bgl*II site engineered in plasmid pRK48. Nucleotide changes are indicated above the bars. Arrows labeled PE1 and PE2 indicate the positions of antisense *bhuR* primers used in primer extension analyses. The dot denotes the transcription initiation site determined with primer PE2. Amino acids of the N-terminal region of the BhuR protein are designated below the nucleotide sequence.

chiseptica, the major site was the upstream adjacent G residue. Consistent with previous predictions (57), the transcription initiation sites were optimally spaced from σ^{70}-like −10 and −35 elements: 5′-TAAAAT-3′ and 5′-TTGCAT-3′, respectively. The initiation sites and promoter elements overlap predicted Fur binding sites, consistent with a promoter occlusion mechanism of Fur repression. The lack of canonical Shine-Dalgarno sequences suggests that the translational efficiency of the *hurI* mRNA may be low.

Genetic and biochemical characterization of *bhuR* promoter determinants. Other ECF σ factors regulating a variety of functions in response to extracytoplasmic signals have been described (34, 37, 45), and these sigma factors recognize promoter sequences distinct from those typical of σ^{70} promoters (16, 37). Nucleotide sequence alignments comparing the *bhuR* upstream region with promoter sequences of other ECF σ factor-regulated genes identified potential −10 and −35 elements that we previously hypothesized to comprise the *bhuR* promoter (Fig. 4) (56). Based on these predictions, an oligonucleotide primer (PE1, Fig. 4) was designed for mapping of the transcription initiation site by primer extension analysis. However, in multiple experiments, a *bhuR*-specific extension product was not produced by PE1 (data not shown). At that time, it was hypothesized that the extremely high G+C content of the predicted *bhuR* initial transcribed region (93% from positions 370 to 410, Fig. 4) may be causing premature termination of reverse transcription.

To genetically test the prediction that sequences located at positions 335 to 346 constituted a critical part of the HurI-dependent, heme-responsive *bhuR* promoter, a block substitution mutation in the predicted −35 region was constructed (Fig. 4) and analyzed in the context of a transcriptional *hurIR bhuR-lacZ* fusion (plasmid pRK47). B013N(pRK47) showed the same pattern of iron-regulated, heme-responsive *bhuR* transcription as cells carrying the wild-type fusion gene (pRK42) (data not shown), indicating that the residues mutated in pRK47 did not constitute part of the heme-responsive *bhuR* promoter.

Since predictions based on nucleotide sequence alignments with other ECF σ factor promoters did not allow identification of the *bhuR* promoter, a series of deletions in the *bhuR* upstream DNA region was constructed to spatially define the minimal region required for maximal heme-responsive *bhuR* promoter activity. It was previously shown that a 0.5-kb region encompassing the 3′ region of *hurR*, the 0.2-kb *hurR-bhuR* intergenic region, and 5′ *bhuR* sequences carried all the regulatory determinants necessary to direct *hurI*-dependent, heme-responsive transcription of a *bhuR-lacZ* fusion (pRK41) in *B. bronchiseptica* (57). Successive 5′ deletions of this region were obtained by PCR, yielding 0.44-kb, 0.38-kb, and 0.3-kb fragments, which were used to construct *bhuR-lacZ* fusion plasmids pRK51, pRK50, and pRK45, respectively (Fig. 4).

B. bronchiseptica B013N carrying the *bhuR-lacZ* fusion plasmids were grown in iron-depleted medium with or without hemin and assayed for β-galactosidase activity (Fig. 5). B013N (pRK41) showed a ninefold induction of *bhuR* transcription when iron-starved cells were exposed to hemin. Cells carrying fusion plasmid pRK51 or pRK50 exhibited essentially equivalent levels of transcriptional activity and induction, indicating that *bhuR* promoter determinants mediating heme responsiveness were contained within the 0.21-kb region upstream of the *bhuR* start codon carried on plasmid pRK50 (Fig. 4). However, B013N(pRK45) showed markedly reduced transcriptional activity under both growth conditions, and induction in response to hemin was reduced to only ≈3-fold (Fig. 5). This result indicated that nucleotide sequences between the pRK50 and pRK45 endpoints (positions 214 and 291, Fig. 4) were required for wild-type levels of *bhuR* promoter activity. The residual activity and partial heme responsiveness of the fusion borne on

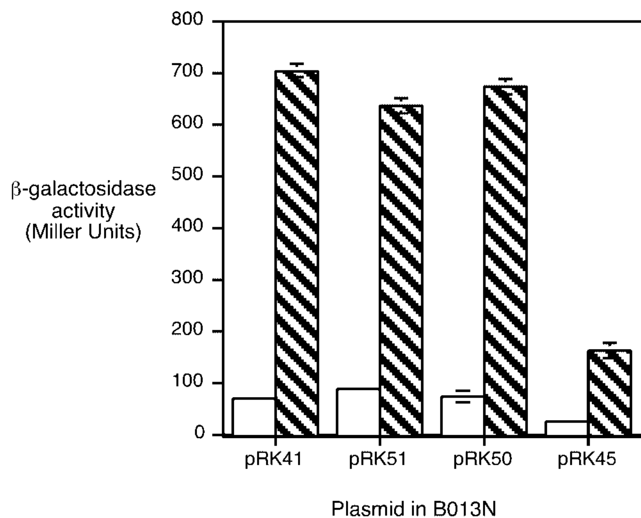

FIG. 5. Determination of the minimal heme-responsive *bhuR* promoter region. *B. bronchiseptica* B013N carrying *bhuR-lacZ* reporter plasmid pRK41, pRK51, pRK50, or pRK45 was cultured in parallel in iron-depleted SS medium with (hatched bars) or without (open bars) hemin supplementation. Bars represent Miller units of LacZ activity ± 1 standard deviation ($n = 3$).

pRK45 suggested that part of the *bhuR* promoter may be contained on this cloned DNA fragment.

Based on these genetic analyses of the *bhuR* promoter, it was hypothesized that the *bhuR* +1 position was located further upstream of the *bhuR* open reading frame than originally predicted and that failure to obtain extension products in previous experiments was perhaps due to the distance between primer PE1 and the transcription initiation site, as well as the G+C composition of sequences upstream of the *bhuR* coding sequences. Additional primer extension analyses performed with primer PE2 (complementary to a region upstream of the high-G+C tract) (Fig. 4) demonstrated the presence of a *bhuR* transcript in RNA samples from iron-starved *B. pertussis* and *B. bronchiseptica* cells that were exposed to hemin (Fig. 6). The transcript was undetectable in RNA samples from iron-replete cultures and was present in very low abundance in iron-starved *B. bronchiseptica* cells (detectable only after extended exposures of the autoradiogram).

The major *bhuR* transcription initiation site corresponded to an A residue 116 nucleotides upstream of the predicted *bhuR* start codon (Fig. 4 and Fig. 6) in both *Bordetella* species. A minor initiation site mapped to a G residue 2 nucleotides further upstream. A larger primer extension product (open arrow, Fig. 6) was detected in RNA samples from iron-starved *B. bronchiseptica* cells (Fig. 6, inset), and was present in very low abundance in RNA from iron-starved *B. bronchiseptica* cells induced with hemin. This primer extension product was also detectable in RNA from iron-starved *B. pertussis* when the autoradiogram was significantly overexposed. This larger product maps to a site 36 nucleotides upstream of the major *bhuR* transcription initiation site and is likely to be derived from a longer iron-regulated transcript initiating upstream of *bhuR*, possibly at the *hurI* promoter. The greater abundance of this

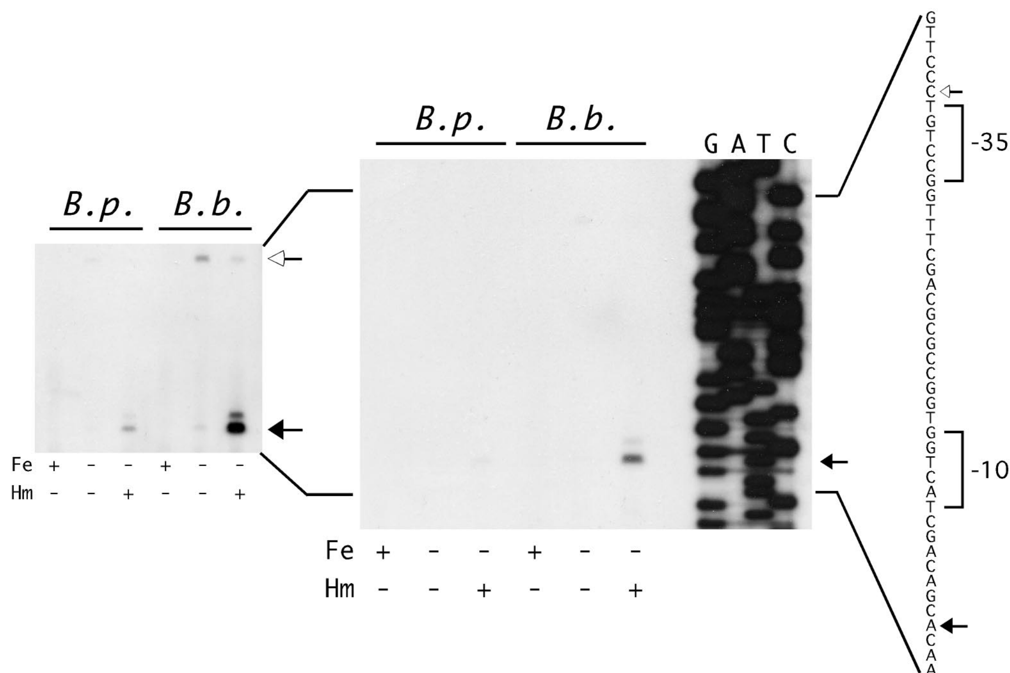

FIG. 6. Mapping the *bhuR* promoter region by primer extension analysis. The autoradiogram at the right shows the results of primer extension with *bhuR*-specific primer PE2 on *B. pertussis* (*B. p.*) and *B. bronchiseptica* (*B. b.*) total RNA from iron-replete cultures (Fe+, Hm−) and iron-depleted cultures with (Fe−, Hm+) or without (Fe−, Hm−) hemin. The inset is an overexposure of the same autoradiogram to show less abundant products. The sequence of the *bhuR* promoter region is shown to the right. The major *bhuR* transcriptional start site is indicated by a solid arrow; the deduced −10 and −35 promoter elements are indicated by brackets. The open arrow upstream of the −35 element indicates the position of the larger iron-regulated product (likely derived from an upstream promoter) in both *B. pertussis* and *B. bronchiseptica* samples.

A

	-35 / -10	
	TCCCtgtccgGTTTCGACGCGCCGGTggtcatCGACAGCACA	*bhuR*
	ACTGTAAGgaaaatAATTCTTATTTCGATTgtccttTTTACCCTT	*fecA*
	GCGTTCCGgaacttTTTGCACGCACGCGAGctctcgAATTTTGCG	*dagA*P2
	CTTGCATTgaacttGTGGATAAAATCACGGtctgatAAAACAGT	*rpoH*P3
	AACGGCCggaactTCCCTCGCAGAGAAAAcatcctATCACCGCGA	*algD*
	CCACGtaaattGCAGGCGATGCCGTTCgttgcaGATCAGG	*pvdF*
	GAGCGCCggaaacACTTTCGCAGGTGGCCcgtagaGGAGTCGGGT	*carQ*

B

Sequence	Gene
GGCAAAAAAAAATTCCTGTTCCCtgtccgGTTTCGACGCGCCGGTggtcatCGACAGCACAAGCACGCTCGTGCCTGCTCTCGAT...ATG	*bhuR*
GCAAAAAAAGTTGCGCAGGGGTggtccgATTTCGCGCCTCACGAgacttaACCCTCAGCACTTCAAATCAATGGGGGAACACTTGTG	*pupB*

FIG. 7. Alignment of ECF σ factor-dependent promoter regions. The promoters of known ECF σ factor-dependent genes are aligned. The transcriptional start site of each gene is underlined. The −10 and −35 promoter elements are indicated by underlined lowercase letters. (A) Genes (GenBank accession numbers): *bhuR, B. pertussis* heme receptor (AY032627); *fecA*, *E. coli* ferric citrate receptor (S79758); *dagA*P2, *Streptomyces coelicolor* agarase promoter 2 (X05811); *rpoH*P3, *E. coli* heat shock σ factor promoter 3 (AF127104); *algD*, *P. aeruginosa* alginate (M28683); *pvdF*, *P. aeruginosa* pyoverdin biosynthesis (U07359); *carQ*, *Myxococcus xanthus* transcriptional regulator (X71062). (B) Alignment of *B. pertussis bhuR* and *P. putida pupB* pseudobactin receptor gene (X73598) promoter regions. Putative −10 and −35 elements are indicated by underlined lowercase letters. The start codons and the *B. pertussis* +1 position are underlined.

larger product in RNA from iron-starved cells in the absence of hemin is similar to the pattern of expression of the *hurI* transcript (Fig. 3), suggesting that this larger primer extension product may be derived from a transcript initiating at the *hurI* promoter. It is possible that termination of reverse transcription may occur at this point on the transcript due to the presence of a secondary structure in the mRNA.

We previously reported that in *B. bronchiseptica*, heme-inducible transcription of a *bhuR-lacZ* fusion was dependent on *hurI* (57). To determine whether *bhuR* transcription was also initiated in a *hurI*-dependent manner in *B. pertussis*, primer extension experiments with RNA obtained from wild-type (UT25Sm1) and Δ*hurI* mutant (PM8) *B. pertussis* strains were performed (data not shown). Similar to the results shown in Fig. 6, in wild-type *B. pertussis* the *bhuR* transcript was most abundant in iron-starved cells exposed to hemin. In contrast, the *B. pertussis* Δ*hurI* mutant showed no detectable *bhuR* transcript under any of the conditions tested (data not shown), indicating that production of the heme-inducible *bhuR* transcript is dependent on the HurI σ factor in *B. pertussis*. The larger iron-regulated product seen in primer extension experiments (such as that shown in Fig. 6) was also observed in other experiments with both wild-type and *hurI* mutant strains (data not shown), indicating that this transcript is not *hurI* dependent.

A nucleotide sequence alignment of the *bhuR* promoter region with other ECF σ factor- dependent promoters is shown in Fig. 7. Consistent with previous observations of other investigators (16, 37), certain features of the ECF σ factor promoters of other organisms, including the −35 elements and spacing between −35 and −10 elements, are fairly well conserved, while the −10 elements are poorly conserved. It has been proposed that the −10 element may provide specificity for promoter recognition by a particular ECF σ factor, since many bacterial genomes appear to encode multiple ECF sigma factors (37, 58). The *bhuR* promoter shows little sequence similarity to other ECF σ factor promoters, even that of *fecA*, which is regulated by another member of the iron starvation subfamily of ECF σ factors (1). Interestingly, although determination of the *P. putida pupB* promoter (regulated by the ECF σ factor PupI) has not been reported, alignment of the *pupB* upstream region with the *bhuR* promoter region revealed striking similarities in what are predicted to be the −35 and −10 elements in each of these promoters. In addition, a tract of A residues upstream from the predicted −35 element is present in the promoter regions of both *bhuR* and *pupB*. The functional significance of this sequence feature, if any, is unknown.

Analysis of transcription through the *hurR-bhuR* intergenic region. The requirement for BhuR in heme-responsive transcriptional activation of the *bhu* genes was demonstrated previously (57), suggesting a role for the receptor as an environmental heme sensor and signal-transducing protein in addition to its function as a heme transporter. Results from the present study indicate that the *bhuR* promoter is active almost exclusively under iron starvation conditions in the presence of heme; however, in order to have BhuR displayed on the cell surface to act as a heme sensor, some transcription of *bhuR* likely occurs under iron starvation conditions in the absence of heme.

To determine if iron-regulated transcription originating at a distal upstream promoter reads through the *hurR-bhuR* intergenic region to contribute to iron-regulated *bhuR* expression, a polar Ω Cm element insertion was constructed in the *hurR-bhuR* intergenic region. Plasmid-borne transcriptional *lacZ* fusions with the wild-type parental *hurIR bhuR′* fragment (pRK42), the fragment containing an engineered *Bgl*II site (pRK48), and the fragment containing the Ω Cm insertion (pRK49) (Table 1, Fig. 8A) were analyzed in wild-type *B. bronchiseptica*.

B013N(pRK42) and B013N(pRK48) exhibited equivalent levels of iron-regulated, heme-inducible *bhuR* expression (Fig. 8B). Levels of β-galactosidase activity were increased by ≈2-fold in response to iron starvation compared with levels in iron-replete cells, and further activated by approximately 4.5-fold by the addition of heme. In contrast, in B013N(pRK49),

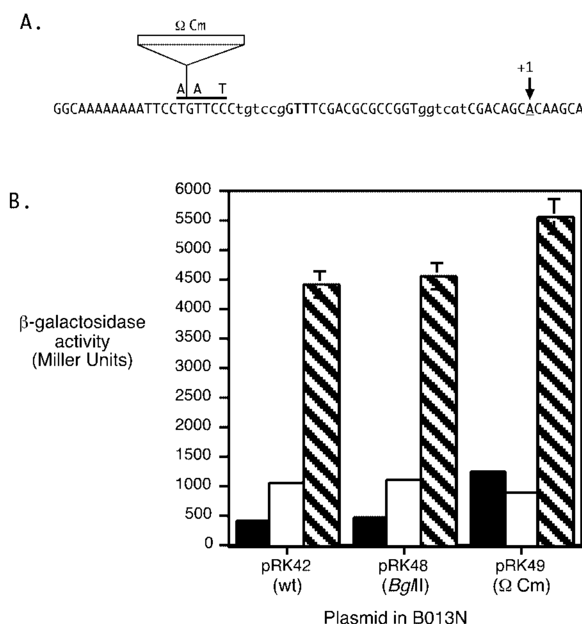

FIG. 8. Analysis of transcriptional readthrough in the *hurR-bhuR* intergenic region. (A) The nucleotide sequence of a portion of the *hurR-bhuR* intergenic region is shown from 5′ to 3′. The first nucleotide corresponds to position 251 in Fig. 4. The solid horizontal bar over the nucleotide sequence indicates the position of the *Bgl*II site; the nucleotide substitutions that created this restriction site are indicated above the bar. The insertion site of the chloramphenicol resistance cassette containing transcriptional terminators on both ends (Ω Cm) is indicated. The −35 and −10 elements of the *bhuR* promoter are indicated in lowercase letters. The major *bhuR* transcriptional initiation site is indicated with an arrow labeled +1. (B) *B. bronchiseptica* B013N cells carrying the designated plasmids were cultured in iron-replete (solid bars), iron-depleted (open bars), or iron-depleted medium with hemin (hatched bars) and assayed for β-galactosidase activity. Bars represent LacZ activity ± 1 standard deviation ($n = 3$). Parental plasmid pRK42 (wt) contains the wild-type *hurIR bhuR-lacZ* transcriptional fusion. Plasmid pRK48 (*Bgl*II) is identical to pRK42 except for three substituted nucleotides (indicated in A) that create a *Bgl*II site. The 3.2-kb Ω Cm cassette was cloned into the *Bgl*II site of pRK48 to construct plasmid pRK49 (Ω Cm).

the β-galactosidase activities of iron-replete and iron-depleted cultures were nearly equivalent, indicating that the insertion abolished iron-regulated *bhuR* expression. However, transcription of *bhuR* was heme activated ≈5.5-fold over iron-depleted levels in B013N(pRK49), demonstrating that the Ω Cm insertion did not disrupt heme-responsive *bhuR* promoter function. These results suggest that transcription resulting in iron-regulated, heme-independent *bhu* gene expression originates upstream of the site of the Ω cassette insertion. Transcription from the *hurI* promoter was shown to be iron regulated, and thus it is likely that transcription from the *hurI* promoter reads through the *hurR-bhuR* intergenic region and into *bhuR* to allow low levels of *bhuR* transcription under iron-limiting conditions in the absence of heme induction.

Additional evidence indicating that iron-regulated transcription through the *hurR-bhuR* intergenic region contributes to *bhuR* expression in the absence of inducer was obtained by reverse transcription-PCR analysis. Total RNA from *B. pertussis* cells grown under iron-replete conditions and iron-depleted conditions with and without hemin supplementation was reverse transcribed, and the products were used as the template in PCR. The predicted 0.44-kb product, encompassing the *'hurR-bhuR'* region, was obtained when cosmid DNA carrying the entire *hur-bhu* genetic system was used as a control template (Fig. 9, lane 1). A *'hurR-bhuR'* transcript was not detected in RNA from cells grown in iron-replete medium (Fig. 9, lane 3), consistent with Fur repression at the *hurI* promoter. In contrast, transcripts spanning the *hurR-bhuR* intergenic region were detected in RNA samples from iron-starved cells cultured with and without hemin (Fig. 9, lanes 5 and 7). These results confirm that RNA transcripts initiating upstream of the heme-inducible *bhuR* promoter (likely at the *hurI* promoter) proceed through the *hurR-bhuR* intergenic region and into *bhuR* under iron-limiting conditions in the absence of inducer, thus allowing BhuR to be produced at a low level for heme sensing and transport.

DISCUSSION

Studies on the *B. pertussis* heme utilization system to date (56, 57) support the model proposed in Fig. 10 for iron-repressible and heme-responsive transcriptional regulation of *bhu* genes. Under iron-replete conditions, Fur and iron repress *hurI* promoter activity (Fig. 10A). Under iron-depleted conditions, Fur derepression of the *hurI* promoter allows transcription initiation at *hurI*, resulting in HurI and HurR protein production. However, in the absence of heme, HurI remains inactive through its association with HurR. Some transcription initiated at the *hurI* promoter reads through the *hurR-bhuR* intergenic region and the *bhu* genes (Fig. 10B), allowing low levels of BhuR to be produced and displayed on the cell surface. When BhuR binds heme, a signal is transduced through HurR, and HurI is released and can associate with core RNA polymerase to direct high levels of transcription at the *bhuR* promoter (Fig. 10C). Transcription at the *hurI* promoter may continue until the cell's intracellular iron stores are replenished, at which time Fur repression will resume. HurI-depen-

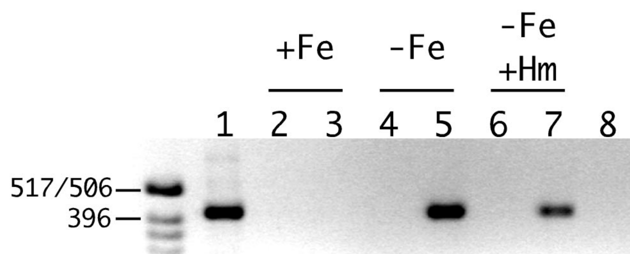

FIG. 9. Reverse transcription-PCR analysis of transcription in the *hurR-bhuR* intergenic region. Total RNA from wild-type *B. pertussis* cells was isolated, reverse transcribed, and used as a template in PCR analysis. The 0.44-kb product encompasses 3′ *hurR* sequences, the *hurR-bhuR* intergenic region, and 5′ *bhuR* sequences. The positive control DNA template was cosmid pCPbhu1 (lane 1); negative control reactions contained no reverse transcriptase (lanes 2, 4, and 6) or RNA template treated with RNase prior to reverse transcription (lane 8). Cells were grown in iron-replete (lanes 2 and 3), iron-depleted (lanes 4, 5, and 8), and iron-depleted with hemin (lanes 6 and 7) medium. The sizes of DNA markers (in base pairs) are indicated at the left. The image is inverted from the ethidium bromide-stained agarose gel photographed under UV transillumination.

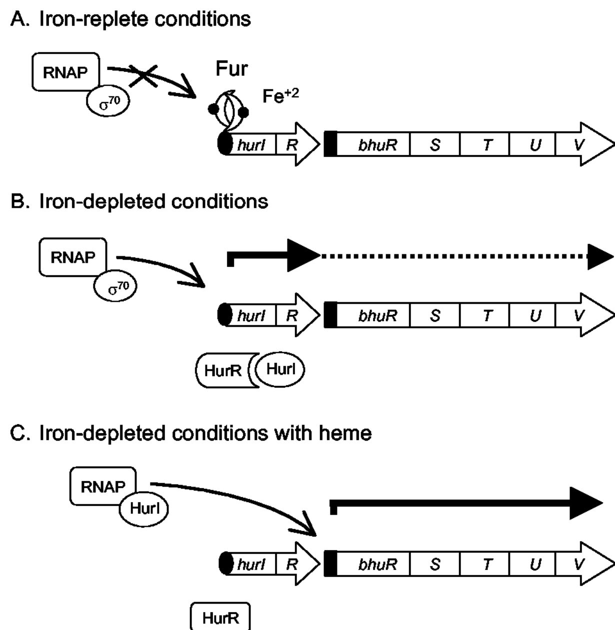

FIG. 10. Model for molecular mechanisms of transcriptional regulation of the *Bordetella* heme iron utilization system. The genetic regulation of *Bordetella* heme utilization genes under three different environmental conditions is depicted as described in the Discussion. The *hur* and *bhu* genes are identified by open arrows, which indicate the direction of transcription. Solid arrows represent transcripts originating at the *hurI* and *bhuR* promoters; the dashed line with a solid arrowhead indicates a putative low-abundance readthrough transcript. Curved arrows point to positions of transcription initiation. The solid oval and solid rectangle indicate the iron-regulated *hurI* promoter and heme-responsive *bhuR* promoter, respectively. RNAP, RNA polymerase core enzyme.

dent transcription of the *bhu* genes may diminish and eventually cease as the HurI protein turns over and no new protein is produced.

The *hurI* and *bhuR* promoters were mapped by mutational and primer extension analyses. The transcription initiation site for the *hurI* gene was consistent with previous predictions of σ^{70}-like promoter elements. Iron regulation of *hurI* was observed, consistent with predicted Fur binding sites and previous determination of functional Fur binding activity in the *hurI* promoter region (56). Several lines of evidence suggest that an iron-regulated polycistronic transcript initiating at the *hurI* promoter and reading through *bhuR* provides a low level of *bhuR* expression in the absence of heme inducer. First, the *hurI* and *hurR* open reading frames overlap, and no other obvious promoter elements exist within these coding regions, suggesting that they are cotranscribed. Additionally, reverse transcription-PCR experiments identified iron-regulated transcripts encompassing the *hurR-bhuR* intergenic region, indicating that readthrough transcription occurs. Concordantly, insertion of a terminator downstream of *hurR* abolished the wild-type pattern of iron-regulated *bhuR* expression but did not affect heme-activated expression (Fig. 8), indicating that the transcript encompassing the *hurR-bhuR* intergenic region was iron regulated and *hurI* independent, which is a pattern of expression identical to that of the *hurI* transcript (Fig. 3). Iron-regulated, heme-independent *bhuR* expression is predicted to be crucial for BhuR production in the absence of inducer, which would allow *B. pertussis* cells to sense the presence of heme in the environment.

The *bhuR* transcription initiation site was identified in both *B. pertussis* and *B. bronchiseptica*. Consistent with our previous studies examining the activity of *bhuR-lacZ* reporter fusions (57), the *bhuR* transcript was found to be iron regulated, heme inducible, and *hurI* dependent. A second, larger product was also identified with a *bhuR*-specific primer in primer extension analyses. This product was iron regulated but not heme responsive or *hurI* dependent; thus, this pattern of expression is very similar to that of the *hurI* transcript. The *bhuR* promoter shares little similarity with characterized ECF σ factor promoters from other organisms, including the *fecA* promoter of *E. coli*, which is regulated by the iron starvation ECF sigma factor FecI. Interestingly, the *bhuR* promoter region shares several features with the predicted *P. putida pupB* promoter region, including the presence of an adenine-rich region upstream of the predicted −35 elements, suggesting that the regulation of these promoters may also be similar.

Though the concentrations of heme to which *Bordetella* cells are exposed in vivo are unknown, the success of another obligate human respiratory pathogen, *Haemophilus influenzae*, implies that heme may be accessed in this niche by capable organisms. Similar to *B. pertussis*, nontypeable *H. influenzae* is a noninvasive organism that colonizes the human nasopharynx. *Haemophilus* species are incapable of synthesizing protoporphyrin IX, the precursor of heme, and require exogenously supplied heme or porphyrin in order to grow aerobically (23). Thus, their ability to successfully colonize the nasopharynx and cause upper respiratory disease in humans indicates that their heme requirements are satisfied in the host environment. Unlike *Haemophilus* species, *B. pertussis* and *B. bronchiseptica* can synthesize heme precursors and thus do not require heme as a growth factor. However, heme internalized via the Bhu system may be used both as an iron source and as a prosthetic group for direct incorporation into cytochromes and other metabolic enzymes.

The *bhu* system is the second example of a positively regulated *Bordetella* iron acquisition system for which the substrate is known. The native alcaligin siderophore system is positively regulated by an AraC-like protein, AlcR, in response to iron starvation and the presence of alcaligin. We hypothesize that positive regulation of iron acquisition systems in *Bordetella* species allows the organisms to prioritize expression of genes based on iron source availability. During the course of infection, it is likely that cells may sense multiple iron sources, for example, heme and ferric alcaligin, simultaneously. Under those circumstances, priority might be assigned to expression of genes that encode utilization functions for the most abundant or most easily assimilated iron source in the environment. The ability to integrate signals received from multiple iron sources and respond appropriately may be critical for *B. pertussis* in the complex host environment, which changes over the course of infection due to the actions of *B. pertussis* virulence factors and the host immune responses.

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