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# Heme-Responsive Transcriptional Activation of *Bordetella bhu* Genes

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***Bordetella pertussis* and *Bordetella bronchiseptica*, gram-negative respiratory pathogens of mammals, possess a heme iron utilization system encoded by the *bhuRSTUV* genes. Preliminary evidence suggested that expression of the BhuR heme receptor was stimulated by the presence of heme under iron-limiting conditions. The *hurIR* (heme uptake regulator) genes were previously identified upstream of the *bhuRSTUV* gene cluster and are predicted to encode homologs of members of the iron starvation subfamily of extracytoplasmic function (ECF) regulators. In this study, *B. pertussis* and *B. bronchiseptica* Δ*hurI* mutants, predicted to lack an ECF σ factor, were constructed and found to be deficient in the utilization of hemin and hemoglobin. Genetic complementation of Δ*hurI* strains with plasmid-borne *hurI* restored wild-type levels of heme utilization. *B. bronchiseptica* Δ*hurI* mutant BRM23 was defective in heme-responsive production of the BhuR heme receptor; *hurI* in *trans* restored heme-inducible BhuR expression to the mutant and resulted in BhuR overproduction. Transcriptional analyses with *bhuR-lacZ* fusion plasmids confirmed that *bhuR* transcription was activated in iron-starved cells in response to heme compounds. Heme-responsive *bhuR* transcription was not observed in mutant BRM23, indicating that *hurI* is required for positive regulation of *bhu* gene expression. Furthermore, *bhuR* was required for heme-inducible *bhu* gene activation, supporting the hypothesis that positive regulation of *bhuRSTUV* occurs by a surface signaling mechanism involving the heme-iron receptor BhuR.**

To overcome iron deficiency in the host environment, bacterial pathogens have evolved high-affinity iron uptake mechanisms (58). Numerous bacterial species utilize siderophores that scavenge extracellular iron and also remove transferrin- and lactoferrin-bound iron (39, 52). Specific cell surface receptors that allow direct removal of iron from host proteins, including transferrin, lactoferrin and hemoproteins, are also produced by some bacteria (27, 46, 62, 71).

The Fur (ferric uptake regulator) protein, with ferrous iron as corepressor, represses transcription of iron uptake genes when intracellular iron concentrations are sufficient (5). In some cases, Fur derepression under iron-limiting conditions is the only requirement for expression of iron-regulated genes; however, certain iron acquisition systems also require positive regulation for full expression of iron transport functions. Three classes of positive regulators controlling expression of iron uptake genes in gram-negative bacteria have been described and include AraC-like regulators (8, 25, 30, 57), two-component signal transduction systems (19, 60), and extracytoplasmic function (ECF) σ factors (2, 10, 37). These regulators activate transcription of iron transport genes in response to iron starvation and the presence of the cognate iron compound.

The *Escherichia coli* ferric citrate uptake (Fec) system is a well-characterized example of an iron transport system that is positively regulated by an ECF σ factor. Transcriptional activation of *fec* genes involves signaling through the FecA outer membrane receptor to the FecR cytoplasmic membrane protein (23, 35). The activity of the ECF σ factor FecI is modulated by an undefined mechanism through direct physical interaction with FecR (23, 45), so that FecI-dependent transcription of *fec* genes occurs only under iron starvation conditions in the presence of the cognate inducer ferric citrate (10).

Host hemoproteins represent an abundant pool of iron (56) that is utilized by some bacterial pathogens. Heme iron utilization systems have been described for a number of gram-negative bacteria, including *Serratia marcescens* (40), *Neisseria* spp. (42, 67), *Pseudomonas aeruginosa* (41, 55), *Yersinia* spp. (66, 69), and *Shigella dysenteriae* (48). The most commonly described system consists of a heme-specific, TonB-dependent outer membrane receptor, a periplasmic heme-binding protein, and an ATP-binding cassette permease which delivers heme to the cytoplasm (27, 71). This type of heme uptake system is negatively regulated at the transcriptional level by iron through the Fur repressor (40, 48, 55, 65, 69).

*Bordetella pertussis* and *Bordetella bronchiseptica* are closely related gram-negative respiratory pathogens of mammals capable of acquiring iron through production and utilization of the native siderophore alcaligin (13, 50), by utilization of non-native siderophores (xenosiderophores) such as enterobactin (7) and ferrichrome (6), and by utilization of host heme compounds (1, 53). We have shown that the *bhuRSTUV* gene cluster encodes functions required for heme iron utilization in *B. pertussis* and *B. bronchiseptica* (70) (Fig. 1). The *bhuR* gene encodes the outer membrane heme receptor, and the other deduced components of the heme system consist of a putative heme utilization factor (BhuS), the periplasmic heme-binding protein (BhuT), a cytoplasmic membrane permease protein (BhuU), and an ATP-binding protein (BhuV). Open reading frames predicted to encode an ECF σ factor, HurI, and cytoplasmic membrane regulator, HurR, were identified immediately upstream of the *B. pertussis* and *B. bronchiseptica* *bhuRSTUV* genes (70). The more distantly related avian respiratory pathogen *Bordetella avium* possesses an apparently orthologous heme utilization system encoded by the *rhuIR* and

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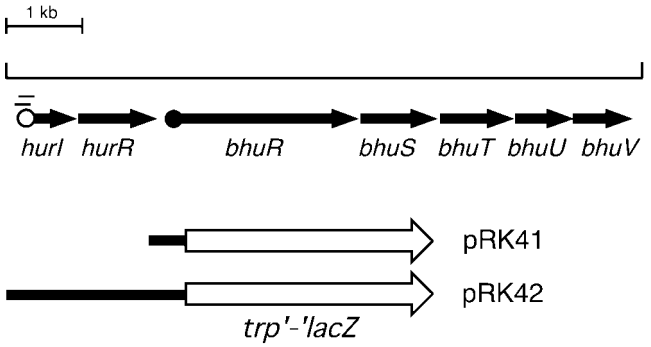


FIG. 1. Features of *Bordetella hur-bhu* genetic system and *bhuR-lacZ* transcriptional fusions. The *bhu* genes encode a heme utilization system in *B. pertussis* and *B. bronchiseptica* (70). The arrows represent the spatial limits and direction of transcription of genes. The open circle represents the predicted $\sigma^{70}$-dependent *hurI* promoter, and vertical bars indicate predicted overlapping Fur-binding sites. The solid circle upstream of *bhuR* indicates the location of sequences similar to ECF σ factor −10 and −35 elements predicted to comprise a HurI-dependent promoter. Transcriptional *bhuR-lacZ* and *hurIR bhuR-lacZ* fusions were constructed and maintained on low-copy plasmids pRK41 and pRK42, respectively. Solid bars represent portions of the *hur-bhu* genetic region, and the open arrow represents the promoterless *trp'-'lacZ* gene.

*bhuRSTUV* genes (51), and expression of *bhuR* was shown to be activated by RhuI, a HurI homolog (36).

The similarity of the *B. pertussis* and *B. bronchiseptica* HurI and HurR proteins to the ECF regulators FecI and FecR of *E. coli*, as well as the observation that production of the BhuR outer membrane heme receptor was heme responsive, led to the hypothesis that the *bhu* genes are positively regulated in response to heme compounds (70). In the present study, we show that the *B. pertussis hurI* gene is required for optimal heme-iron utilization as well as heme-responsive *bhuR* transcription and BhuR protein production. Furthermore, we found that *bhuR* is required for heme-inducible *bhuR* transcription. These results, along with similarities between the *Bordetella* heme utilization systems and the *E. coli* Fec system, support a model of iron-repressible, heme-inducible *bhu* gene transcription.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** *Bordetella* strains and recombinant plasmids are listed in Table 1. Plasmid vector pGEM3Z (Promega, Madison, Wis.) was used in routine cloning procedures, and suicide plasmid vector pSS1129 (64) was used to construct mutant *Bordetella* strains. Broad-host-range plasmids pRK415 (≈5 to 8 copies/cell) (34) and pBBR1MCS (≈30 copies/cell) (3, 38) were used in the construction of plasmids for complementation of *Bordetella* mutant strains or to carry reporter gene fusions.

**Bacterial culture conditions.** *E. coli* strains were grown in Luria-Bertani (LB) broth or on LB agar plates. *B. pertussis* and *B. bronchiseptica* strains were cultured at 37°C on Bordet Gengou (BG) agar (9) and LB agar, respectively. Stainer-Scholte (SS) broth (63), modified as described previously (61), was used for growth of *Bordetella* strains in defined liquid medium. Iron-depleted SS medium was prepared by treatment with Chelex100 (Bio-Rad, Richmond, Calif.) as described previously (4) and contained no iron supplements; iron-replete SS broth contained 36 μM $FeSO_4$. Stock solutions of bovine hemin chloride (Sigma, St. Louis, Mo.) and human hemoglobin (Sigma) were prepared as described previously (70) and added to liquid cultures at final concentrations of 5 μM and 1.25 μM, respectively, unless otherwise indicated. The media used to culture *B. bronchiseptica* and *B. pertussis* for growth stimulation bioassays were LB agar and modified LB agar (Pertussis LB), respectively (70). Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 30 μg/ml; gentamicin, 10 μg/ml; kanamycin, 50 μg/ml; streptomycin, 50 μg/ml; and tetracycline, 15 μg/ml (for *B. bronchiseptica* and *E. coli*) or 10 μg/ml (for *B. pertussis*).

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

| Strain or plasmid | Description | Reference or source |
|---|---|---|
| UT25Sml | *B. pertussis*; spontaneous streptomycin-resistant derivative of wild-type strain UT25 | 12 |
| PM8 | *B. pertussis* UT25Sml Δ*hurI* | This study |
| B013N | *B. bronchiseptica*; spontaneous nalidixic acid-resistant derivative of wild-type B013 | 4 |
| BRM21 | B013N *bhuR*Ωp3Z77; BhuR$^-$; heme utilization deficient; Kan$^r$ Amp$^r$ | 70 |
| BRM23 | *B. bronchiseptica* B013N Δ*hurI* | This study |
| pRK37 | pRK415 carrying 1.3-kb *B. pertussis* UT25 *hurI* gene and predicted promoter region; Tet$^r$ | This study |
| pRK40 | pRK415 with 3.3-kb *Eco*RI-*Hin*dIII *trp'-'lacZ* insert fragment; Tet$^r$ | This study |
| pRK41 | pRK40 with 0.5-kb *B. pertussis* UT25 *'hurR bhuR'* insert fragment; *bhuR-lacZ* transcriptional fusion; Tet$^r$ | This study |
| pRK42 | pRK40 with 2.1-kb *B. pertussis* UT25 *hurIR bhuR'* insert fragment; *hurIR bhuR-lacZ* transcriptional fusion; Tet$^r$ | This study |
| pBB29 | pBBR1MCS with 8-kb *Xho*I-*Sal*I fragment containing *B. pertussis* UT25 *'hurIR bhuRSTUV*; Chl$^r$ | This study |
| pBB33 | pBBR1MCS carrying 1.3-kb *Bam*HI-*Hin*dIII *B. pertussis* UT25 *hurI* fragment subcloned from pRK37; Chl$^r$ | This study |
| pBB34 | pBBR1MCS with 4.3-kb *Hin*dIII-*Kpn*I fragment containing *B. pertussis* UT25 *'hurIR bhuRS'*; Chl$^r$ | This study |

**Genetic methods.** General cloning procedures were performed with host strain *E. coli* DH5α (Invitrogen, Carlsbad, Calif.). Triparental matings for the transfer of plasmids from DH5α donors to *Bordetella* recipients have been described previously (12); mobilization functions were provided by DH5α harboring plasmid pRK2013 (26).

The *hurI* nucleotide sequence of *B. pertussis* UT25 was determined on both DNA strands. Nucleotide sequencing was performed by the Advanced Genetic Analysis Center at the University of Minnesota. Oligonucleotide primers were synthesized by Invitrogen or Integrated DNA Technologies (Coralville, Iowa). Nucleotide and protein sequence data were analyzed as described previously (70). Other nucleotide sequence data were from the *B. pertussis* Tohama I or *B. bronchiseptica* RB50 genome sequences produced by the *Bordetella* Sequencing Groups (http://www.sanger.ac.uk/Projects/B_pertussis and http://www.sanger.ac.uk/Projects/B_bronchiseptica) at the Sanger Centre. Servers from the Sanger Centre and the National Center for Biotechnology Information (NCBI) at the National Library of Medicine were used for database searches.

Southern hybridization analysis of *B. pertussis* genomic DNA from wild-type and candidate *hurI* mutant strains was performed as described previously (59). The *hurI*-specific DNA hybridization probe was radiolabeled with [α-$^{32}$P]dCTP (ICN Radiochemicals, Irvine, Calif.) by the random priming method (24) with the Random Primers DNA labeling system (Invitrogen).

PCR was used to amplify *B. pertussis* DNA regions, including the *hurI* gene, the presumptive *bhuR* promoter region (GenBank accession number AY032627) identified by similarity to other ECF σ factor promoters (22, 49), and the *hurIR bhuR'* DNA fragment. PCR primers included appropriate adapters containing restriction enzyme sites to facilitate cloning. PCRs were carried out with *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, Calif.) essentially as described previously (11). The DNA template for all PCR amplifications was 300 ng of

cosmid pCPbhu1 DNA (70), containing the entire *B. pertussis hurIR bhuRSTUV* genetic region.

The promoterless *lacZ* gene used to construct *bhuR-lacZ* transcriptional fusions was derived from mini-Tn*5 lacZ1* (20). The *trp′-′lacZ* fragment was cloned as a 3.3-kb *Eco*RI-*Hin*dIII DNA fragment to the broad-host-range plasmid pRK415, resulting in plasmid pRK40 (Table 1). A 0.5-kb fragment containing the predicted *bhuR* promoter region, spanning the 3′ end of *hurR*, the *hurR-bhuR* intergenic region, and the 5′ *bhuR* region, was PCR amplified with primers containing *Eco*RI adapter ends. This 0.5-kb *bhuR* promoter region was cloned to the *Eco*RI site upstream of the *trp′-′lacZ* cassette in pRK40 to create the *bhuR-lacZ* transcriptional fusion plasmid pRK41. A 2.1-kb fragment containing *hurIR* and the 5′ *bhuR* region was PCR amplified with primers containing *Eco*RI adapters and similarly cloned to plasmid pRK40 to construct *hurIR bhuR-lacZ* fusion plasmid pRK42. The same antisense *bhuR* primer was used in PCRs to amplify the 0.5-kb *bhuR* promoter and the 2.1-kb *hurIR bhuR′* fragment used for construction of reporter plasmids pRK41 and pRK42, respectively, so that the *bhuR-lacZ* fusion junctions were identical.

**Construction of *Bordetella* Δ*hurI* mutant strains.** An in-frame deletion of 297 bp of *hurI* coding sequence was constructed by PCR overlap extension as described previously (11, 32). Sequences flanking the desired *hurI* deletion were PCR amplified with primer pair Hur1 (5′-GGGGGATCCGTTCGCGCTCAC AATGTC-3′) and Hur2 (5′-GAACGCCTGGCGCACTTTTTGCAGCCAGCC GTGATG-3′) and primer pair Hur3 (5′-CATCACGGCTGGCTGCAAAAAG TGCGCCAGGCGTTC-3′) and Hur4 (5′-GGGGAATTCGGTCGGCTTCGA GGTAGA-3′). The Hur2 and Hur3 primers were designed so that the Hur1 and Hur2 and the Hur3 and Hur4 PCR products had complementary 21-nucleotide overlaps at the termini (spanning the region to be deleted). These PCR products were combined at a 1:1 molar ratio, denatured, and annealed, and the Δ*hurI* fragment was amplified with the outside primers Hur1 and Hur4 which carried restriction enzyme site adapters for cloning of the PCR product to allelic exchange vector pSS1129. The resulting plasmid, pSS9, was mated to *B. pertussis* strain UT25Sm1, and the Δ*hurI* mutation was transferred to the *B. pertussis* chromosome by homologous recombination as described by Stibitz (64) to produce Δ*hurI* mutant PM8. Allelic exchange was verified by PCR and by Southern hybridization analysis with a *hurI*-specific DNA probe (data not shown).

To construct a *B. bronchiseptica* Δ*hurI* mutant by allelic exchange, plasmid pSS9 was delivered to *B. bronchiseptica* B013N by electroporation, and plasmid integrants were selected based on ampicillin and gentamicin resistance. These integrants were passaged without antibiotic selection for several generations to allow the second crossover needed for allelic exchange. Passaged cells were plated for isolated colonies and then replicated onto LB agar and LB agar containing gentamicin to score for loss of pSS9-derived gentamicin resistance. Gentamicin-sensitive strain BRM23 was determined by PCR analysis to contain the correct Δ*hurI* mutation (data not shown).

**Hemin and hemoglobin growth stimulation bioassays.** Growth stimulation bioassays of hemin and hemoglobin utilization by *B. pertussis* and *B. bronchiseptica* strains were performed as described previously (13, 70). Images of bioassay plates were obtained with an AGFA Arcus II flatbed scanner.

**Heme-responsive BhuR protein production.** *B. bronchiseptica* strains B013N (wild type), BRM21 (*bhuR*Ωp3Z77) (70), BRM23 (Δ*hurI*), and BRM23 (pRK37) (Δ*hurI*/*hurI*$^+$) were grown on LB agar for 24 h and inoculated to iron-replete SS broth (with tetracycline at a final concentration of 15 μg/ml for BRM23 [pRK37]). Cells were grown for 24 h with shaking and then subcultured 1:200 to one iron-replete and two iron-depleted SS cultures. After 18 h of growth, hemin was added to one iron-depleted culture at a final concentration of 1.25 μM, and all cultures were grown for 4 additional h. Cells were harvested, and the total membrane fractions were prepared and analyzed as described previously (33). Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels and visualized by Coomassie blue staining.

**β-Galactosidase assays.** *B. bronchiseptica* strains carrying low-copy-number *bhuR-lacZ* reporter plasmid pRK41 or pRK42 or control plasmid vector pRK40 were grown in iron-replete SS medium and then subcultured as described for BhuR protein production. Eighteen hours after subculture, hemin was added to one iron-depleted culture at a final concentration of 5 μM. The iron-replete and both iron-depleted cultures were grown for 8 additional h and assayed for β-galactosidase activity by the method of Miller (47), as modified by Brickman and coworkers (15). β-Galactosidase activities represent the means of triplicate assays ($n = 3 \pm 1$ standard deviation). The results reported are representative of at least two separate experimental trials.

**Nucleotide sequence accession number.** The GenBank accession number for the *B. pertussis* UT25 *hurI* gene is AF508979.

## RESULTS

**Determination of *B. pertussis* UT25 *hurI* nucleotide sequence.** To analyze *hurI* and its role in *bhu* gene regulation and to examine potential differences between the sequenced *B. pertussis* Tohama I strain and our clinical isolate UT25, the nucleotide sequence of the UT25 *hurI* gene was determined. The UT25 *hurI* open reading frame encodes a 169-amino-acid protein with a molecular mass of 19.3 kDa. A TBLASTN search at the NCBI database confirmed that the HurI protein is similar to members of the ECF family of σ factors. The UT25 HurI protein displays 41% and 46% similarity to the FecI and PupI σ factors of *E. coli* and *Pseudomonas putida*, respectively (GenBank accession numbers: FecI, M63115; PupI, X77918). The *B. pertussis* UT25 *hurI* coding sequence is 100% identical to that of *B. pertussis* Tohama I *hurI*, 99% identical to the *B. bronchiseptica* RB50 *hurI* gene, and 72% identical to the *B. avium rhuI* gene (GenBank accession number AY095952). *B. pertussis* and *B. bronchiseptica* HurI amino acid sequences are 100% identical, while the *B. avium* RhuI protein shows 69% similarity to HurI.

Upstream of the UT25 *hurI* gene, putative −10 (5′-TAAA AT-3′) and −35 (5′-TTGCAT-3′) regions similar to the *E. coli* $\sigma^{70}$ promoter consensus sequence (29) were identified. Overlapping inverted repeat sequences with 67% and 73% identity to the consensus Fur-binding sequence (16, 21) were found in the predicted −10 region. This observation is consistent with previous experiments that demonstrated strong functional Fur binding in this region (70), suggesting that *hurI* expression is iron regulated.

**Construction and phenotypic analysis of *Bordetella hurI* mutants.** To determine the role of *hurI* in heme utilization, Δ*hurI* mutant strains PM8 (*B. pertussis*) and BRM23 (*B. bronchiseptica*) were constructed. An in-frame deletion derivative of the *B. pertussis hurI* gene which lacked 297 bp internal to the *hurI* coding sequence was constructed by PCR overlap extension. The resulting altered HurI protein would lack a 99-amino-acid sequence that includes σ factor regions predicted to be involved in core RNA polymerase binding and −10 promoter sequence recognition (44). *Bordetella* Δ*hurI* mutants PM8 and BRM23 were tested for the ability to use hemin and hemoglobin in growth stimulation bioassays.

The wild-type *B. bronchiseptica* strain, B013N, was capable of utilizing both hemin and hemoglobin as iron sources, exhibiting well-defined, dense zones of growth surrounding wells containing either compound (Fig. 2). In contrast, isogenic *B. bronchiseptica* Δ*hurI* mutant BRM23 was significantly less proficient in utilization of hemin and hemoglobin, as demonstrated by weak, diffuse zones of growth. Genetic complementation of BRM23 with the *hurI*$^+$ plasmid pRK37 restored a wild-type heme utilization phenotype (Fig. 2), indicating that the BRM23 heme utilization defect was due to the mutation in *hurI*. BRM23 harboring plasmid vector control pRK415 remained defective in heme utilization (data not shown). A similar defect in heme utilization was observed in *B. pertussis* Δ*hurI* mutant strain PM8, and the mutant phenotype was restored to wild type by *hurI*$^+$ plasmid pRK37 (data not shown). These bioassay results indicate that *hurI* encodes a product that is required for optimal utilization of heme iron compounds by *B. bronchiseptica* and *B. pertussis.* The phenotypes of


FIG. 2. Heme utilization by *B. bronchiseptica* strains. Growth stimulation bioassays with solutions of bovine hemin chloride or human hemoglobin added to wells: A, 50 μM hemin; B, 100 μM hemin; C, 25 μM hemoglobin. Strains: B013N, wild-type *B. bronchiseptica* parent strain; BRM23, Δ*hurI*; BRM23(pRK37), BRM23 carrying plasmid-borne *hurI*.

the Δ*hurI* mutant strains are consistent with the hypothesis that *hurI* encodes a positive regulator which functions to increase expression of heme transport genes.

**Analysis of *hurI*-dependent BhuR production.** Heme-responsive BhuR protein production was previously observed in wild-type *B. bronchiseptica* strain B013N (70). To determine if this increased BhuR production in response to hemin is mediated by *hurI*, BhuR levels in total membrane fractions from wild-type and mutant strains were examined by SDS-PAGE after growth in hemin-containing media. *B. bronchiseptica* strains B013N (wild type), BRM21 (*bhuR*), BRM23 (Δ*hurI*), and BRM23(pRK37) (Δ*hurI*/*hurI*$^+$) were grown in iron-replete SS medium or iron-depleted SS medium with or without hemin supplementation. None of the cells grown in iron-replete medium appeared to produce proteins of the predicted 90-kDa molecular mass of BhuR (Fig. 3). Cells grown in iron-depleted medium without hemin supplementation produced multiple iron-regulated membrane proteins in the 80- to 97-kDa molecular mass range, making resolution of the BhuR protein difficult. However, BhuR production was observed in BRM23(pRK37) cells carrying the *hurI* gene on a low-copy-number plasmid (Fig. 3, lane 8).

Wild-type *B. bronchiseptica* B013N showed a pattern of BhuR production consistent with previous results (70): BhuR was present in low abundance in the membrane fractions of iron-starved wild-type cells (Fig. 3, lane 5), while iron-starved cells exposed to hemin produced significant levels of BhuR (Fig. 3, lane 9). As expected, the *bhuR* mutant BRM21 failed to produce BhuR protein under any growth condition tested (Fig. 3, lanes 2, 6, and 10). Similarly, BhuR was not detected in membrane fractions of the Δ*hurI* mutant strain BRM23 (Fig. 3, lanes 3, 7, and 11). In contrast, BRM23 cells carrying the *hurI*$^+$ plasmid pRK37 produced some BhuR under low-iron growth conditions and significantly elevated levels when starved for iron and exposed to hemin (Fig. 3, compare lanes 8 and 12).

These data indicate that production of BhuR is iron re-

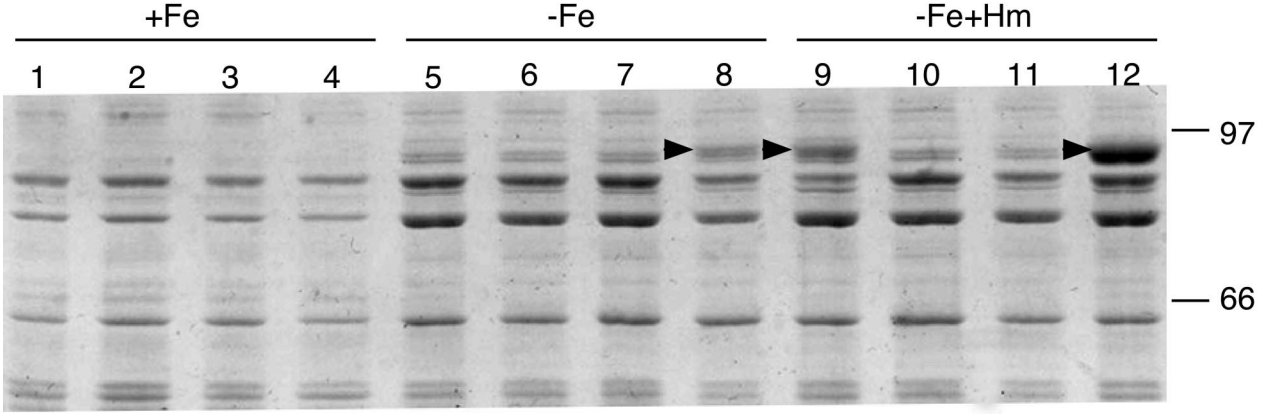


FIG. 3. *hurI*-dependent, heme-responsive BhuR protein production. Total envelope proteins from *B. bronchiseptica* cell fractions were separated by SDS-PAGE. Arrows indicate the migration position of the BhuR protein. Samples are grouped according to the culture conditions used: +Fe, iron-replete; −Fe, iron-depleted; −Fe+Hm, iron-depleted with hemin added to a 1.25 μM final concentration after 18 h of growth. Strains: B013N, wild-type parent strain (lanes 1, 5, and 9); BRM21, *bhuR* (lanes 2, 6, and 10); BRM23, Δ*hurI* (lanes 3, 7, and 11); BRM23(pRK37), BRM23 carrying plasmid-borne *hurI* (lanes 4, 8, and 12).

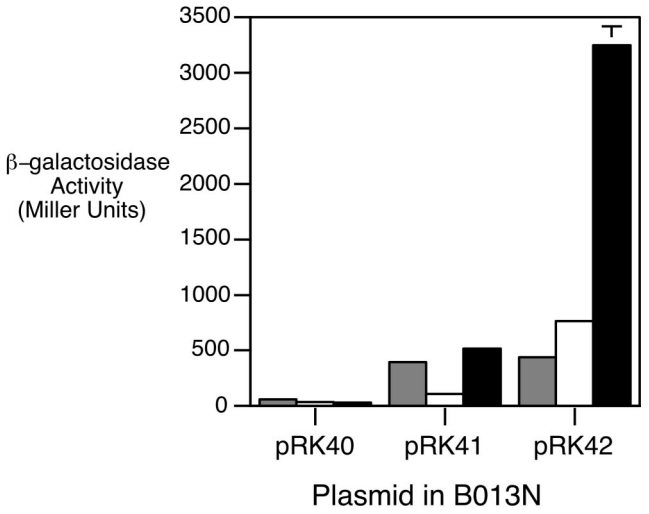


FIG. 4. Heme-regulated *bhuR* transcription in wild-type *B. bronchiseptica*. Bacteria were grown in iron-replete or iron-depleted SS broth with or without hemin and assayed for β-galactosidase activity as described in Materials and Methods. Bars represent β-galactosidase activities of B013N carrying the plasmid vector control (pRK40), *bhuR-lacZ* (pRK41), or *hurIR bhuR-lacZ* (pRK42) reporter fusion plasmid. Gray bars, iron-replete; open bars, iron-depleted; solid bars, iron-depleted with hemin supplementation.

pressed, *hurI* dependent, and heme inducible. Furthermore, the BhuR overproduction phenotype in BRM23(pRK37) cells carrying five to eight copies of *hurI* is consistent with the proposed function of HurI as a regulator that directs transcription from the *bhuR* promoter.

***bhuR* transcription is heme responsive.** The HurI protein is predicted to be an ECF σ factor which directs transcription from a promoter upstream of *bhuR* when iron-starved cells are exposed to heme compounds. Since the *hur-bhu* systems of *B. pertussis* and *B. bronchiseptica* are nearly identical at the nucleotide sequence level and BhuR production was heme responsive and *hurI* dependent in *B. bronchiseptica* (70) (Fig. 3), analyses of *B. pertussis bhuR* promoter activity were facilitated by using *bhuR-lacZ* transcriptional fusions (Fig. 1) in the more readily cultivated species *B. bronchiseptica*.

Strains were grown in iron-replete SS medium and iron-depleted SS medium with or without hemin. Wild-type *B. bronchiseptica* B013N cells carrying the control plasmid vector pRK40 exhibited low levels of β-galactosidase activity under all growth conditions tested (Fig. 4). Iron-replete B013N cells carrying the *bhuR-lacZ* fusion (pRK41) produced approximately 400 Miller units of β-galactosidase activity, while the *bhuR* transcriptional activity of the iron-depleted strain was nearly fourfold lower. This pattern of expression is similar to that of the positively regulated *Bordetella* alcaligin siderophore system: in the absence of alcaligin inducer, iron-starved cultures exhibit lower *alcABCDER* transcriptional activity than parallel iron-replete cultures (11, 14). However, supplying iron-starved B013N(pRK41) with 5 μM hemin resulted in a fivefold increase in β-galactosidase activity over that of iron-starved cells that were not exposed to hemin. Iron starvation was a prerequisite for heme-inducible *bhuR* expression, since cells grown in iron-replete SS broth did not exhibit elevated *bhuR* transcriptional activity in response to hemin (data not shown).

The *hur* and *bhu* genes are transcribed in the same direction, and some *bhu*-specific transcripts may actually initiate at the predicted upstream iron-regulated *hurI* promoter. Although cells carrying the pRK41 *bhuR-lacZ* fusion plasmid exhibited heme-inducible transcription under iron-limiting conditions, it was reasoned that removal of the *bhuR* promoter region from the natural genetic context of the *hurIR bhuRSTUV* gene cluster may alter *bhuR* transcription. Therefore, another reporter fusion plasmid which carries *hurIR bhuR-lacZ* (pRK42) (Fig. 1) was constructed and analyzed in wild-type strain B013N (Fig. 4). B013N(pRK42) cells demonstrated iron-repressible and heme-inducible expression of *bhuR*, which correlated with the results from the BhuR protein analyses (Fig. 3). Iron starvation resulted in a twofold increase in *bhuR* transcriptional activity compared with that of iron-replete cells. Transcription of *bhuR-lacZ* was further increased fourfold upon addition of heme to iron-depleted cells. Iron-replete B013N(pRK42) cells did not exhibit enhanced *bhuR-lacZ* expression in the presence of hemin (data not shown), indicating that both iron starvation and the presence of heme are required for optimal *bhuR* expression.

**Heme-responsive *bhuR* transcription is *hurI* dependent.** To determine whether heme-responsive *bhuR* transcription is mediated by the putative ECF σ-factor HurI, *bhuR* promoter activity was monitored in *B. bronchiseptica* Δ*hurI* mutant BRM23. BRM23 cells carrying the plasmid-borne *bhuR-lacZ* fusion (pRK41) were grown in medium with or without added hemin. Iron-replete cultures of Δ*hurI* strain BRM23 exhibited levels of *bhuR-lacZ* transcriptional activity similar to those of wild-type B013N (data not shown). Iron-depleted BRM23 (pRK41) cells yielded negligible levels of β-galactosidase activity, and *bhuR* transcription was not enhanced in response to hemin, indicating that *hurI* is required for heme-responsive *bhuR* transcription (Fig. 5A). Complementation of the BRM23 Δ*hurI* mutant with plasmid pRK42 carrying *hurIR* in *cis* to the *bhuR-lacZ* reporter fusion restored heme-responsive *bhuR* transcriptional activation: iron-starved BRM23(pRK42) cells exposed to hemin exhibited a fourfold increase in β-galactosidase activity over the level in iron-starved cells grown in the absence of hemin. These results are consistent with the BhuR protein studies (Fig. 3), in which BRM23 complemented with *hurI* in *trans* showed increased levels of BhuR production compared with Δ*hurI* mutant BRM23. These data demonstrate that heme-inducible *bhuR* transcriptional activation is absolutely dependent on *hurI*.

**BhuR is required for heme-responsive *bhuR* transcriptional activation.** The signaling process leading to transcriptional activation of the *fecABCDE* genes in *E. coli* has been shown to require the ferric citrate outer membrane receptor protein FecA (28). Recognition of ferric citrate by FecA results in signaling through its N-terminal domain to the FecR cytoplasmic membrane protein and subsequently to release or activation of the FecI σ factor for transcription of the *fec* genes. The BhuR N terminus is highly similar to that of FecA (70), suggesting that BhuR may be involved in a similar signaling cascade resulting in transcriptional activation of *bhu* genes in response to heme inducer.

To examine the effect of a *bhuR* mutation on the transcrip-

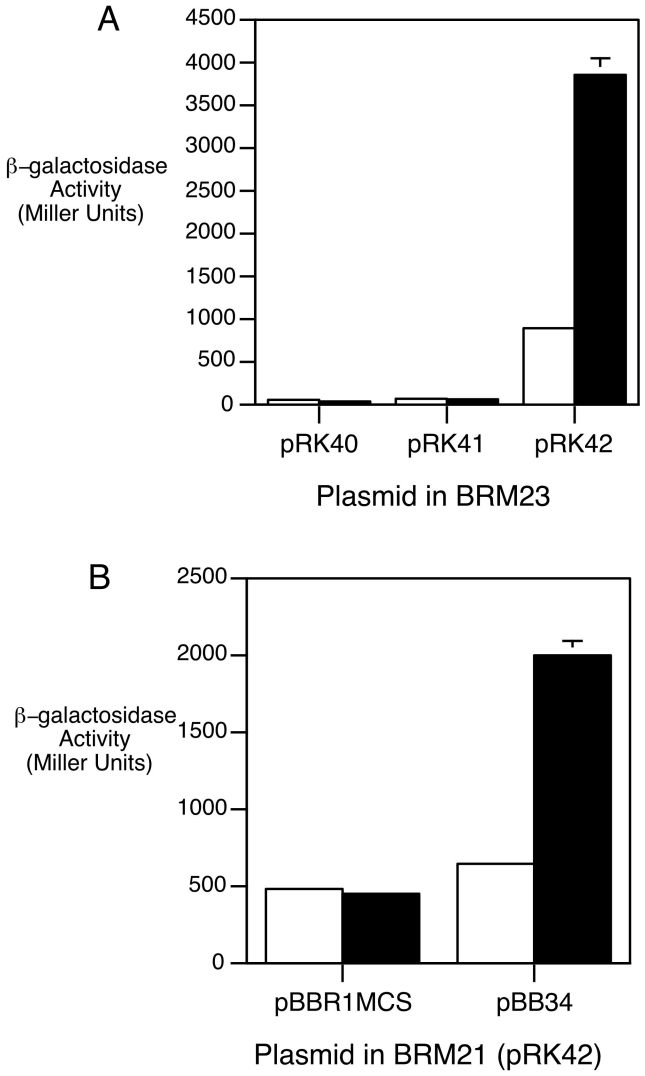


FIG. 5. Heme-responsive *bhuR* transcription is *hurI* and *bhuR* dependent. *B. bronchiseptica* strains were cultured as described in Materials and Methods. Bars indicate β-galactosidase activities of cultures grown under different conditions: open bars, iron-depleted; solid bars, iron-depleted with hemin supplementation. (A) Strain BRM23 (Δ*hurI*) carrying plasmid vector control pRK40, *bhuR-lacZ* reporter plasmid pRK41, or complementing *hurIR bhuR-lacZ* reporter plasmid pRK42. (B) Strain BRM21 (*bhuR*) carrying pRK42 and vector control pBBR1MCS or *bhuR*$^+$ plasmid pBB34.

tional responsiveness of the *bhuR* promoter, *bhuR-lacZ* transcriptional activity was examined in *B. bronchiseptica bhuR* mutant strain BRM21 (Fig. 5B). Iron-starved BRM21 cells carrying the *hurIR bhuR-lacZ* fusion plasmid pRK42 along with a compatible plasmid vector control, pBBR1MCS, failed to show heme-inducible *bhuR* expression. However, plasmid pBB34 (*bhuR*$^+$) restored heme-inducible *bhuR* transcription to BRM21(pRK42), indicating that BhuR is required for heme-responsive transcriptional activation. The chromosomal *bhuR* mutation of BRM21 results in a defect in heme iron utilization and is also polar on the downstream *bhuSTUV* genes (70). Heme iron utilization was not fully restored to BRM21 by *bhuR*$^+$ plasmid pBB34 but was restored to wild-type levels by plasmid pBB29, carrying *bhuRSTUV* (data not shown). These results indicate that BhuR is involved in the heme-signaling cascade resulting in *bhuR* transcriptional activation but that transport of heme or *bhuSTUV*-encoded functions may not be required for this process.

## DISCUSSION

To initiate and sustain a productive infection, *Bordetella* species must acquire iron in the iron-restricted host environment. Production and utilization of the native siderophore alcaligin (13, 50) and utilization of xenosiderophores that may be present on host mucosa may allow *B. pertussis* and *B. bronchiseptica* to scavenge iron. Host molecules, including transferrin, lactoferrin, and heme compounds, also represent potential sources of iron that could be accessed during colonization and persistence within a host. The *bhu* genetic system of *B. pertussis* and *B. bronchiseptica* was identified and demonstrated to be required for heme utilization (70).

Heme iron utilization systems similar to the *Bordetella bhu* system have been described for a number of gram-negative bacterial pathogens, including *Vibrio cholerae* (54), *Yersinia pestis* (69), *Yersinia enterocolitica* (65), and *P. aeruginosa* (55). The genes encoding heme transport systems in these organisms are negatively regulated by iron through the Fur repressor; however, no apparent Fur-binding activity was detected in the *B. pertussis bhuR* promoter region (70), which suggested that the mechanism of *Bordetella bhu* gene regulation may differ from that of other characterized systems. BhuR protein was not produced in substantial amounts under iron-limiting conditions, but it was produced in much greater abundance when iron-starved cultures were exposed to hemin. The identification of the *hurIR* genes immediately upstream of the *bhu* gene cluster led to the hypothesis that the *bhu* system is positively regulated under iron-limiting conditions in the presence of the cognate substrate, heme.

Based upon similarities in gene organization and predicted amino acid sequences between the *Bordetella hur-bhu* system and the well-characterized *E. coli fec* system, along with results from the present study, we propose a model for regulation of the *Bordetella bhu* genes. Under iron starvation conditions, Fur derepression of the promoter upstream of *hurI* would allow $\sigma^{70}$-directed transcription of the *hurIR* genes and perhaps low-level expression of *bhuRSTUV* by readthrough transcription. Higher levels of heme-responsive *bhuRSTUV* transcription are predicted to originate from a HurI-dependent promoter upstream of *bhuR.* As proposed for FecI and FecR, HurI would remain inactive through its interaction with HurR until the BhuR outer membrane receptor binds heme, transferring the receptor occupancy signal through HurR to the HurI σ factor, allowing it to activate transcription at the HurI-dependent promoter upstream of the *bhu* genes. In the present study, using two different *bhuR-lacZ* reporter plasmids, we showed that *bhuR* expression is enhanced four- to fivefold when iron-starved cells are exposed to heme. Furthermore, the BhuR heme receptor is required for *hurI*-dependent *bhuR* transcription, indicating its role in the signaling cascade.

*B. avium* possesses a gene cluster, *rhuIR bhuRSTUV* (GenBank accession number AY095952), that is required for heme utilization and is similar to the *B. pertussis* and *B. bronchisep-*

*tica hurIR bhuRSTUV* gene clusters (51). The *rhuI* gene is predicted to encode an ECF σ factor which is 69% identical to HurI. *B. avium* carrying *rhuI* in multicopy showed increased BhuR production and increased expression of a chromosomal *bhuR*::*phoA* fusion under both iron-replete and iron-depleted culture conditions, suggesting that RhuI activates expression of the *B. avium bhu* genes. Similar to our findings in *B. bronchiseptica*, expression of a plasmid-borne *bhuR-lacZYA* fusion in *B. avium* was enhanced by heme compounds, and this responsiveness was BhuR dependent (36). A requirement for RhuI in heme utilization and heme-dependent *bhu* transcription in *B. avium* has not been reported.

*B. pertussis* and *B. bronchiseptica* Δ*hurI* mutants were defective in utilizing heme iron sources in growth stimulation bioassays; the zones of growth were much less turbid and their perimeters were less defined than those of the wild-type parental strains. Perhaps the loss of *hurI* affects the kinetics of heme uptake or efficiency of heme iron utilization due to the low abundance of BhuR receptor on the cell surface. In the model proposed for *bhu* gene regulation, low-level HurI-independent *bhuR* expression in the absence of inducer would be required in order to have some BhuR present at the cell surface to sense the availability of heme in the environment. If this were the case, basal levels of BhuR produced in the absence of *bhu* gene activation (e.g., in a *hurI* mutant) may be sufficient to supply modest growth-promoting levels of heme to the cells. HurI-dependent *bhu* gene activation may be more critical in environments where heme concentrations are much lower and efficient scavenging of heme iron is essential for cell growth.

BhuR expression analysis and *bhuR-lacZ* reporter fusions demonstrated that heme-responsive *bhuR* expression is *hurI* dependent. Cells carrying *hurI* on low-copy-number plasmids overproduced the BhuR protein and exhibited higher levels of *bhuR-lacZ* transcription than wild-type cells carrying a single copy of *hurI.* Differences in levels of *bhuR-lacZ* expression in iron-replete compared with iron-depleted cultures were correlated with the presence or absence of the *hurIR* genes carried in *cis* to the *bhuR-lacZ* fusion. Iron-starved wild-type cells carrying the *hurIR bhuR-lacZ* fusion plasmid pRK42 exhibited approximately twofold more β-galactosidase activity than iron-replete cultures. In contrast, iron-starved wild-type cells carrying *bhuR-lacZ* plasmid pRK41 showed a fourfold reduction in activity compared with iron-replete cultures. It is possible that transcription from the iron-regulated *hurI* promoter on pRK42 reads through *bhuR-lacZ*, resulting in higher levels of expression under iron-limiting conditions. Furthermore, alterations in the stoichiometry between the HurI and HurR regulators and *bhuR* promoter targets could have a major influence on *bhuR-lacZ* expression.

The loss of heme-responsive *bhuR* transcription in *B. bronchiseptica* Δ*hurI* mutant BRM23 demonstrated that HurI is required for *bhuR* transcriptional activation. Interestingly, although complementation of BRM23 (*bhuR-lacZ*) cells with compatible, medium-copy-number (≈30 copies/cell) *hurI*$^+$ plasmid pBB33 restored iron-regulated *bhuR-lacZ* expression, transcriptional activation did not occur in response to hemin (data not shown). If HurR acts as an anti-σ factor that sequesters or inactivates HurI in the absence of heme, excessive levels of HurI production in cells carrying pBB33 might overwhelm the negative influence of HurR and thus result in the observed inducer-independent transcription initiation at the *bhuR* promoter. The *pup* system of *P. putida* WCS358 is regulated by the PupI σ factor and PupR regulator in a manner analogous to the *E. coli* Fec system (37), in that transcription of pseudobactin siderophore uptake genes is *pupI* dependent and pseudobactin responsive. Similar to the heme-independent expression of *bhuR-lacZ* in BRM23 carrying ≈30 copies of *hurI, P. putida pupI* mutants genetically complemented by the *pupI* gene in multicopy show a pattern of inducer-independent *pup* gene transcription (37).

Experiments analyzing *bhuR-lacZ* expression in *B. bronchiseptica bhuR* mutant BRM21 demonstrated that the BhuR outer membrane receptor itself is required for signaling, resulting in heme-responsive *bhu* gene transcriptional activation, but that heme uptake may not be required for this process. The transport and signaling processes are also separable in the *E. coli* Fec system, as evidenced by *fecA* mutants which retain the ability to transport ferric citrate but no longer respond to its presence (28). The extended N-terminal regions of FecA and the *P. putida* PupB receptors are critical for inducer-responsive signaling resulting in *fec* (35) and *pup* (37) transcriptional activation, respectively. This N-terminal region is unique to outer membrane receptors whose expression is regulated by ECF σ factors (10). The BhuR N terminus is highly similar to the corresponding regions of FecA and PupB (70), suggesting similar functional signaling properties among these receptors.

Innate iron restriction in the mammalian host would be expected to lead to derepression of Fur-regulated promoters, resulting in uninduced basal levels of expression of all *Bordetella* iron acquisition systems. Siderophore-mediated iron transport may be most critical during early stages of infection, when organisms must colonize an intact mucosal surface which may not provide a ready source of heme iron. However, if colonization is successful, the elaborated *Bordetella* toxins, including pertussis toxin (for *B. pertussis*) (68), dermonecrotic toxin (43), tracheal cytotoxin (18), and adenylate cyclase/hemolysin (17, 31), would presumably damage the epithelium and other host cells, providing a source of heme iron for *Bordetella* cells. Priority regulation of *Bordetella* iron acquisition systems according to iron source availability may allow faster adaptation to a changing host environment and thus a greater degree of pathogenic success.

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