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The *Bordetella bhu* Locus Is Required for Heme Iron Utilization

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***Bordetella pertussis* and *Bordetella bronchiseptica* are capable of obtaining iron from hemin and hemoglobin. Genes encoding a putative bacterial heme iron acquisition system (*bhu*, for *Bordetella* heme utilization) were identified in a *B. pertussis* genomic sequence database, and the corresponding DNA was isolated from a virulent strain of *B. pertussis*. A *B. pertussis bhuR* mutant, predicted to lack the heme outer membrane receptor, was generated by allelic exchange. In contrast to the wild-type strain, *bhuR* mutant PM5 was incapable of acquiring iron from hemin and hemoglobin; genetic complementation of PM5 with the cloned *bhuRSTUV* genes restored heme utilization to wild-type levels. In parallel studies, *B. bronchiseptica bhu* sequences were also identified and a *B. bronchiseptica bhuR* mutant was constructed and confirmed to be defective in heme iron acquisition. The wild-type *B. bronchiseptica* parent strain grown under low-iron conditions produced the presumptive BhuR protein, which was absent in the *bhuR* mutant. Furthermore, production of BhuR by iron-starved *B. bronchiseptica* was markedly enhanced by culture in hemin-supplemented medium, suggesting that these organisms sense and respond to heme in the environment. Analysis of the genetic region upstream of the *bhu* cluster identified open reading frames predicted to encode homologs of the *Escherichia coli* ferric citrate uptake regulators FecI and FecR. These putative *Bordetella* regulators may mediate heme-responsive positive transcriptional control of the *bhu* genes.**

Pathogenic microorganisms encounter severe iron limitation in mammalian hosts, where the concentration of free iron is several orders of magnitude less than that required to support microbial growth (18). Iron in serum and on mucosal surfaces is sequestered by the host iron binding proteins transferrin and lactoferrin, respectively, while the majority of host iron is found intracellularly in the form of heme and hemoproteins (58). To overcome iron sequestration by the host, pathogenic bacteria have evolved two general types of high-affinity iron acquisition systems that enable them to scavenge iron. In siderophore-dependent microbial iron acquisition systems, high-affinity iron-chelating siderophores are excreted and utilized to obtain nutritional iron (45, 54), while siderophore-independent systems employ cell surface proteins that mediate the direct binding and utilization of host-derived iron compounds (31, 50, 65, 76).

Many gram-negative pathogens use siderophore-independent systems to acquire iron from heme and hemoglobin (31, 76), and expression of the systems studied to date is negatively regulated at the transcriptional level by the ferric uptake regulator (Fur) protein, with ferrous iron as the corepressor (35, 57, 74). One type of heme utilization system described for *Serratia marcescens* (46) and *Pseudomonas* spp. (39, 47) relies on the secretion of small hemophore proteins, which bind heme and deliver it to heme-hemophore-specific outer membrane receptors. A second general mechanism of heme iron acquisition is exemplified by that of certain *Neisseria* spp. which express a bipartite hemoglobin receptor consisting of a TonB-dependent outer membrane receptor component and an accessory outer membrane lipoprotein (20, 48). Yet a third class of heme iron utilization system identified in organisms including *Pseudomonas aeruginosa* (57), *Yersinia* spp. (72, 74), and *Shigella dysenteriae* (51) utilizes a single-component TonB-dependent outer membrane receptor specific for heme, hemoglobin, or other heme compounds.

Bordetella pertussis and *Bordetella bronchiseptica* are gram-negative respiratory pathogens of mammals. In response to iron starvation, they produce the macrocyclic dihydroxamate siderophore alcaligin (15, 53) and also use a variety of heterologous siderophores, including enterobactin (7), ferrichrome, and desferrioxamine B (6), for iron retrieval. These organisms can also obtain iron from host sources transferrin (60, 61), lactoferrin (61), heme (1, 55), and hemoglobin (55).

In this study, we identified a cluster of *B. pertussis* genes (designated *bhu*, for *Bordetella* heme utilization) predicted to encode proteins highly similar to those of bacterial heme iron acquisition systems with single-component TonB-dependent outer membrane receptors. Mutational and phenotypic analyses confirmed that these *Bordetella* genes were required for acquisition of iron from hemin and hemoglobin in *B. pertussis* as well as in the closely related species *B. bronchiseptica*. Nucleotide sequence analysis of the region immediately upstream of the heme utilization gene cluster identified two open reading frames predicted to encode homologs of the *Escherichia coli* ferric citrate uptake system positive regulators FecI and FecR (11), suggesting that a similar positive regulatory mechanism may exist for the *Bordetella* heme system.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* DH5α (Gibco-BRL, Gaithersburg, Md.) was used as the host strain for general cloning procedures and as the donor strain in triparental matings. DH5α harboring plasmid pRK2013 (30)

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provided mobilization functions in triparental matings. *E. coli* reporter strain H1717 (*fhuF-lacZ aroB*), used for Fur repressor titration assays, has been described previously (71). *B. bronchiseptica* B013N (4) and a spontaneous streptomycin-resistant derivative of wild-type *B. pertussis* UT25 (29), UT25Sm1 (14), have also been described previously.

E. coli strains were grown in Luria-Bertani (LB) broth or on LB agar plates. *B. pertussis* and *B. bronchiseptica* strains were cultured on Bordet-Gengou agar (9) and LB agar, respectively. Stainer-Scholte (SS) broth (68), modified as described previously (64), was used for growth of *Bordetella* strains in defined liquid medium. For iron-depleted cultures, SS basal medium was deferrated by treatment with Chelex100 (Bio-Rad, Richmond, Calif.) as described previously (4); iron-replete SS medium contained 36 μM $FeSO_4$, and iron-depleted SS medium contained no iron supplements. Growth of *Bordetella* liquid cultures was monitored using a Klett-Summerson colorimeter equipped with a no. 54 filter (Klett Manufacturing Co., Long Island City, N.Y.). The medium used to culture *B. pertussis* for growth stimulation bioassays was modified LB agar (pertussis LB [PLB] agar), which was LB broth supplemented with 0.12% Molecusol MB cyclodextrin (Pharmatec, Inc., Alachua, Fla.) and 0.15% bovine serum albumin (BSA) (Sigma, St. Louis, Mo.) and solidified with Noble agar (Difco Laboratories, Detroit, Mich.). Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; gentamicin, 10 μg/ml; kanamycin, 50 μg/ml; streptomycin, 50 μg/ml; tetracycline, 15 (for *B. bronchiseptica* and *E. coli*) or 10 μg/ml (for *B. pertussis*).

Plasmids and genetic methods. Plasmid cloning vectors pGEM3Z (Promega, Madison, Wis.) and pRK415 (42) were used in the construction of recombinant plasmids. Plasmid pBSL86 (2) was the source of the kanamycin resistance cassette used in the construction of *B. pertussis* Δ*bhuR*::kan mutant strain PM5. The cosmid-based *B. pertussis* UT25 genomic DNA library has been described previously (17). Suicide vector pSS1129 (69) was used for allelic exchange in the construction of *B. pertussis* mutant PM5. Conjugal transfer of plasmids from *E. coli* donors to *Bordetella* recipients was accomplished as described previously (14).

Nucleotide sequence data were accessed from the incomplete and unannotated *B. pertussis* Tohama I genome sequence. These sequence data were produced by the *Bordetella pertussis* Sequencing Group at the Sanger Centre and can be obtained from http://www.sanger.ac.uk/Projects/B_pertussis. The incomplete genomic sequence of *B. bronchiseptica* strain RB50 was also accessed from the Sanger Centre (http://www.sanger.ac.uk/Projects/B_bronchiseptica). Nucleotide sequences determined in this laboratory were derived from cloned DNA of *B. pertussis* strain UT25 by primer walking on both DNA strands. Nucleotide sequencing services were provided by the Advanced Genetic Analysis Center at the University of Minnesota. Oligonucleotide primers used in sequencing were synthesized by Gibco-BRL. Management and analysis of nucleotide and protein sequence data were performed with the Lasergene sequence analysis software package for the Macintosh PowerPC computer (DNASTAR, Inc., Madison, Wis.). Database searches were accomplished using the BLAST (3) servers provided by the Sanger Centre and the National Center for Biotechnology Information (NCBI) at the National Library of Medicine. The deduced BhuR amino acid sequence was analyzed for the presence of conserved patterns using the Conserved Domain Database and Search Service analysis (reverse position-specific BLAST) algorithm at the NCBI. Putative *B. pertussis* Fur-binding sequences were identified by using the MegAlign module of the Lasergene program to locate DNA regions of at least 50% identity over a 30-nucleotide (nt) region with the proposed consensus *E. coli* Fur-binding site 5′-GATAATGAT AATCATTATC-3′ (19, 24). Amino acid sequence alignments were performed by the CLUSTAL (38) or Jotun-Hein (34) method using the MegAlign software module. The putative BhuR signal sequence cleavage site was predicted using the SignalP server at the Center for Biological Sequence Analysis (http://www.cbs.dtu.dk/services/SignalP/index.html).

The Fur repressor titration assay (71) was used to test the DNA regions upstream of *hurI* and *bhuR* for functional Fur-binding sites. *E. coli* indicator strain H1717 (*fhuF-lacZ aroB*) carrying the *hurI* or *bhuR* upstream DNA regions subcloned to pGEM3Z was plated on lactose MacConkey agar supplemented with 40 μM ferrous ammonium sulfate and appropriate antibiotics. Strain H1717(pGEM3Z) was the negative control, and the positive control was H1717 carrying p3ZFBS (T. J. Brickman, unpublished data), which contains a cloned copy of the *E. coli* consensus Fur-binding DNA sequence (19, 24).

Southern and in situ DNA hybridizations were performed at high stringency as described previously (62). Oligonucleotide probes for in situ DNA hybridizations (Hem1, 5′-GCAAGGACGAAAACACCGGCC-3′; Hem2, 5′-CTGGTAGGTC AACGATACGCG-3′) were synthesized by Gibco-BRL and end-labeled with [γ^{32}-P]ATP (ICN Radiochemicals, Costa Mesa, Calif.) using T4 polynucleotide kinase as described previously (62). *bhuR*-specific DNA hybridization probes and the 1.2-kb *Hin*cII kanamycin resistance cassette were radiolabeled with [α^{32}-P]dCTP (ICN) by the random-priming method (28) using the Random Primers DNA-labeling system (Gibco-BRL).

Construction of *Bordetella bhuR* mutants. A 2.4-kb *Eco*RV DNA fragment encompassing the 3′ region of *bhuR* and 5′ region of *bhuS* was subcloned from *B. pertussis* UT25 recombinant cosmid pCPbhu1 carrying *bhu* sequences to produce plasmid p3Z75 (Fig. 1). This plasmid was digested with *Sma*I and religated, resulting in the deletion of a 1.2-kb *Sma*I DNA region internal to the putative *bhuR* coding region. This deletion derivative, p3Z76, was linearized with *Sma*I and ligated with a 1.2-kb *Hin*cII DNA fragment containing the kanamycin resistance cassette from pBSL86 (2), resulting in Δ*bhuR*::kan plasmid p3Z77 (Fig. 1). A 2.5- kb fragment of p3Z77 encompassing the mutated *bhuR* region was excised using plasmid vector *Eco*RI and *Bam*HI sites and ligated to suicide vector pSS1129. The resulting plasmid, pSS8, was mated to *B. pertussis* strain UT25Sm1, and the mutation was transferred to the chromosome by homologous recombination as described by Stibitz (69) to produce Δ*bhuR*::kan mutant PM5. Allelic exchange in *B. pertussis* mutant PM5 was verified by Southern hybridization using *bhuR*- and kanamycin resistance cassette-specific DNA probes.

A *B. bronchiseptica bhuR* mutant was constructed by delivery of Δ*bhuR*::kan plasmid p3Z77 (Fig. 1), which cannot replicate in *Bordetella* strains, to *B. bronchiseptica* strain B013N by electroporation. Transformants with the plasmid integrated into the chromosome were selected on agar medium containing kanamycin and ampicillin. Southern hybridization using DNA probes specific for *bhuR* and the kanamycin resistance gene was used to confirm the insertion of the plasmid into the *B. bronchiseptica bhuR* locus in mutant strain BRM21.

Hemin and hemoglobin growth stimulation bioassays. Aqueous stock solutions of bovine hemin chloride (Sigma) at concentrations of 1 or 10 mM were made in 0.02 N NaOH. Bovine (Becton-Dickinson, Cockeysville, Md.) and human, pig, turkey, and rabbit (Sigma) hemoglobin stock solutions were prepared in 10 mM HEPES buffer, pH 7.4. Concentrations of hemoglobin stock solutions were confirmed using a plasma hemoglobin diagnostic kit (Sigma) and were adjusted to 3 or 1 mg/ml. Complexes of hemin-BSA (1:1 molar ratio) at 100 μM and of human hemoglobin-haptoglobin (hemoglobin concentration, 100 μM) were prepared by methods described previously (10). Alcaligin was purified as the deferrisiderophore as described by Brickman and coworkers (15) and used as a positive control at an aqueous concentration of 50 μM. Distilled-water diluent was used as the negative control in the bioassays.

For growth stimulation bioassays, *B. pertussis* strains were cultured on PLB agar plates for 3 days. PLB agar plate growth was suspended in deferrated SS basal medium to an optical density (600 nm) of 2.0; 200 μl of this suspension was seeded into 25 ml of molten PLB agar (at 50°C) containing 50 μg of ethylenediamine-di-[(*o*-hydroxyphenyl)acetic acid] (EDDA)/ml and poured into a 90-mm-diameter petri dish. Wells were punched in the seeded solidified agar, and 50-μl volumes of the test solutions were added. The diameters of the growth stimulation zones around wells containing the specified iron source were measured after 60 h of incubation at 37°C. *B. bronchiseptica* bioassays on iron-restricted medium were performed as described previously using LB agar supplemented with EDDA (15). Growth stimulation is reported as the mean diameter from three replicate bioassays and is representative of four experimental trials.

Analysis of hemin-responsive protein expression. *B. bronchiseptica* strain B013N and *bhuR* mutant BRM21 were grown on LB agar at 37°C for 24 h and used to inoculate iron-replete SS broth cultures to an initial density of 15 Klett units. For each strain, the SS culture was grown with shaking at 37°C for 24 h, at which time cells were harvested, washed with SS basal medium, and used to inoculate one iron-replete and two iron-depleted SS cultures. After 15 h of growth, the iron starvation status of the iron-depleted cultures was confirmed by measuring the production of alcaligin using the chrome-azurol S universal siderophore detection assay (66). One of the iron-depleted cultures was supplemented with hemin to a final concentration of 5 μM, and the cultures were allowed to continue growing. All cultures were sampled at 1, 4, and 8 h after the addition of hemin. Cell samples were disrupted using a French press (American Instrument Company, Silver Spring, Md.), and the insoluble total-membrane fractions were prepared as described previously (41). Proteins were treated in solubilization buffer at 100°C for 6 min and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels containing 0.5 M urea as described previously (64); approximately 30 μg of protein was applied to each gel lane, and proteins were visualized by Coomassie blue staining.

Nucleotide sequence accession number. The GenBank accession number assigned to the 871-nt *B. pertussis* UT25 *hurR-bhuR* region is AY032627.

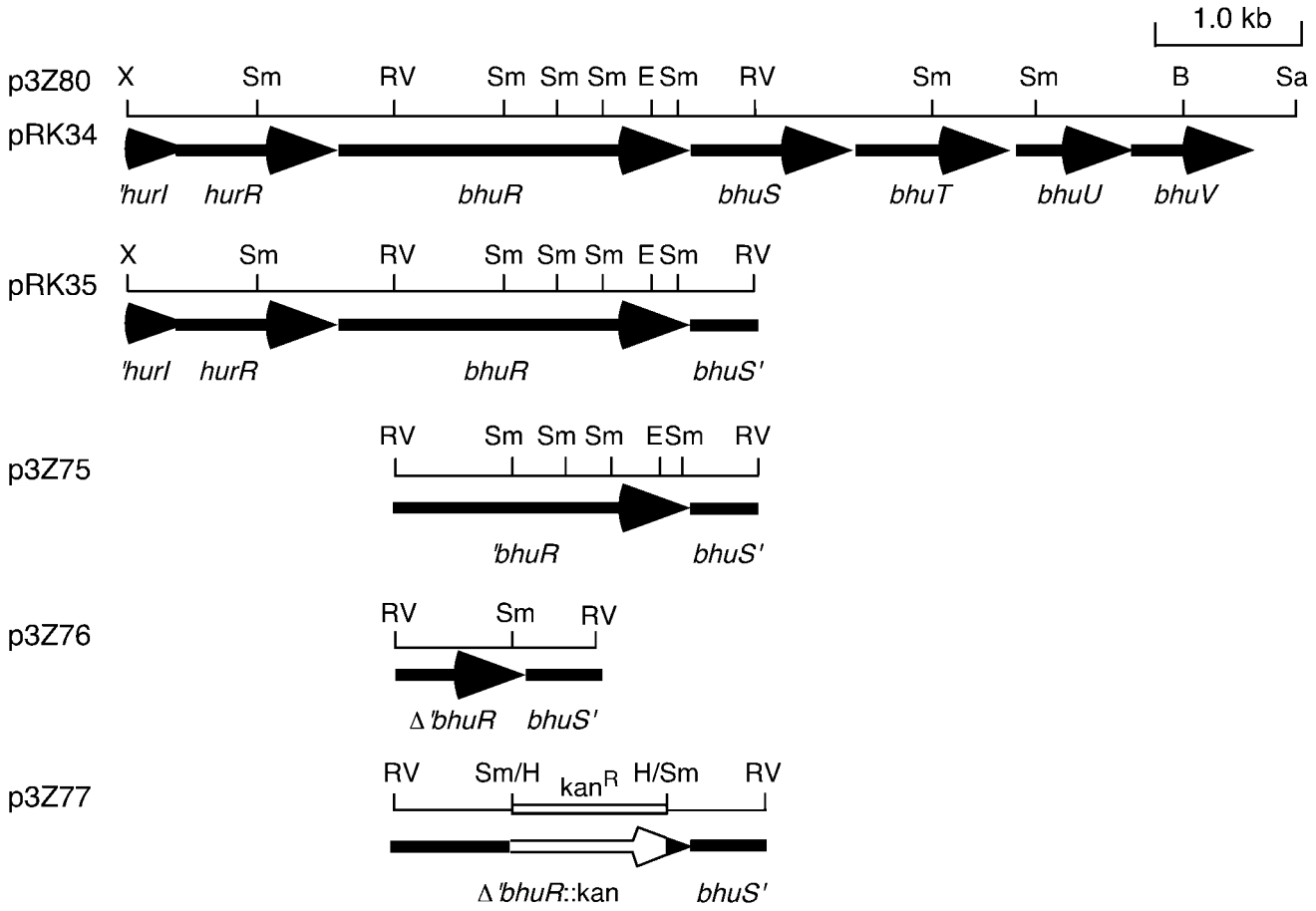

FIG. 1. Spatial organization of the *B. pertussis bhu* genetic regions used to construct recombinant plasmids. The construction of the cosmid subclones and *B. pertussis* Δ*bhuR*::kan mutant PM5 is described in Materials and Methods. Solid arrows, open reading frames (arrowheads denote the direction of transcription); open arrow (DNA insert of plasmid p3Z77), kanamycin resistance gene. The open reading frames upstream of *bhuR* (initially termed *orfI* and *orfR*) are designated *hurIR,* for heme uptake regulators. Abbreviations: B, *Bam*HI; E, *Eco*RI; RV, *Eco*RV; H, *Hin*cII; Sa, *Sal*I; Sm, *Sma*I; X, *Xho*I.

RESULTS

Identification of putative heme utilization genes. To identify *B. pertussis* DNA sequences potentially encoding a heme acquisition system, the incomplete Sanger Centre *B. pertussis* genomic sequence assembly was subjected to a TBLASTN search using the amino acid sequence of the *P. aeruginosa* PhuR heme receptor as the query. PhuR was selected as a representative TonB-dependent heme outer membrane receptor, in part because a BLASTP database search revealed that it was highly similar to heme receptors of several gram-negative bacterial species (57). Although the Sanger Centre *B. pertussis* genomic sequence is presently at the "finishing/gap closure" stage and is thus considered preliminary, the search identified a contig with a 5′-truncated open reading frame (*bhuR*) predicted to encode a protein with significant amino acid sequence similarity to PhuR (Table 1). Downstream of *bhuR* were four closely spaced open reading frames which, based on similarity to components of the *Pseudomonas* Phu system (57), are predicted to encode a hemin-degrading factor (BhuS), a hemin-specific periplasmic binding protein (BhuT), a cytoplasmic membrane permease (BhuU), and an ATP-binding protein (BhuV) (Table 1). The *bhuRSTUV* region of the *B. pertussis* contig was used to search the translated-nucleotide sequence database at the NCBI. The predicted BhuRSTUV proteins were highly similar to proteins of heme utilization systems of several gram-negative organisms, including *Yersinia pestis* (74), *Yersinia enterocolitica* (72), and *S. dysenteriae* (51), as well as *P. aeruginosa* (57) (Table 1). In addition, the organization of the *B. pertussis bhu* open reading frames was very similar to the organization of heme utilization genes in *Yersinia* spp. and *P. aeruginosa*. A search of the Sanger Centre's incomplete genomic sequence database for taxonomically related strain *B. bronchiseptica* RB50 identified homologous DNA sequences having substantial identity with the *B. pertussis bhu* sequences.

Oligonucleotides Hem1 and Hem2, corresponding to *B. pertussis* internal *bhuR* DNA sequences, were used to identify five recombinant cosmids in a *B. pertussis* UT25 genomic library by colony hybridization. Cosmid pCPbhu1 was chosen for limited nucleotide sequencing using the Hem1 oligonucleotide primer to establish the presence of the predicted *bhu* sequences. The results confirmed that the pCPbhu1 DNA sequence was identical to the contig sequence from the Sanger Centre database over the *bhuR* region sequenced (data not shown). Further,

TABLE 1. Characteristics of *B. pertussis* proteins predicted to be involved in heme utilization

B. pertussis protein	Deduced molecular mass (kDa)	Predicted function	Homologous proteins (% similarity)[a]
HurI	19	Transcriptional activator	FecI (44), PupI (46)
HurR	35	Sensor/regulator	FecR (27), PupR (31)
BhuR	92	Outer membrane heme receptor	PhuR (28), PfhR (28), HutA (26), ChuA (26), ShuA (26), HmbR (26)
BhuS	38	Heme-degrading protein	PhuS (46), HemS (41), HmuS (41), ShuS (38)
BhuT	30	Periplasmic binding protein	PhuT (25), HemT (36), HmuT (36), ShuT (35)
BhuU	26	Membrane permease	PhuU (60), HemU (52), HmuU (53), ShuU (50)
BhuV	28	ATP-binding protein	PhuV (50), HemV (40), HmuV (39), ShuV (38)

[a] Percent similarities were determined by the Jotun-Hein method with the PAM250 residue weight table using the MegAlign application of the Lasergene software package. Heme utilization proteins are from the following organisms (GenBank accession numbers are in parentheses): Phu, *P. aeruginosa* (AF055999); PfhR, *P. fluorescens* (AF127222); HutA, *V. cholerae* (L27149); ChuA, *E. coli* (U67920); Shu, *S. dysenteriae* (U64516); HmbR, *N. meningitidis* (AF105339); Hem, *Y. enterocolitica* (X68147 and X77867); Hmu, *Y. pestis* (U60647). FecI and FecR, ferric citrate uptake regulators of *E. coli* (M63115); PupI and PupR, regulators of the *pupB* receptor gene for transport of ferric pseudobactins BN7 and BN8 in *P. putida* WCS358 (X77918).

restriction enzyme mapping of pCPbhu1 concurred with maps deduced from the contig nucleotide sequence, confirming that the cloned *B. pertussis* UT25 DNA encompassed the desired *bhu* region.

Construction and phenotypic analysis of *B. pertussis bhuR* mutant PM5. To determine if the *bhu* genes encoded a functional *B. pertussis* heme iron acquisition system, *bhuR* heme receptor mutant PM5 was constructed (Fig. 1). The ability of isogenic wild-type and Δ*bhuR*::kan mutant strains of *B. pertussis* to use hemin and hemoglobin as iron sources was assessed in growth stimulation bioassays. Wild-type parental strain UT25Sm1 was capable of utilizing hemin and human hemoglobin as sources of iron in these bioassays, exhibiting dose-responsive growth stimulation in response to increasing concentrations of these iron compounds (Table 2). In contrast, Δ*bhuR*::kan mutant PM5 was unable to use either iron source at any concentration tested. Both wild-type and *bhuR* mutant strains formed similar growth haloes around wells containing positive control alcaligin, indicating that alcaligin siderophore-mediated iron utilization was unaffected by the *bhuR* mutation.

In genetic complementation experiments, plasmid pRK34, which carries the *B. pertussis* UT25 8-kb *bhuRSTUV* DNA insert (Fig. 1), restored the ability of mutant PM5 to obtain iron from both hemin and hemoglobin to wild-type levels (Table 2). A smaller subclone containing *bhuR* (pRK35; Fig. 1) did not restore heme iron utilization to PM5 (data not shown),

TABLE 2. Growth stimulation of *B. pertussis* strains by hemin and human hemoglobin

Strain[a]	Diam of growth zone[b] (mm) surrounding wells containing the indicated concn (μM) of iron source:							
	Hemin			Hemoglobin				Alcaligin, 50
	25	50	100	0.8	1.6	4	8	
UT25Sm1	15	17	20	19	21	22	25	35
PM5	—	—	—	—	—	—	—	36
PM5(pRK34)	15	18	23	19	22	23	25	34
PM5(pRK415)	—	—	—	—	—	—	—	36

[a] UT25Sm1, *B. pertussis bhu*$^+$ strain; PM5, UT25Sm1 Δ*bhuR*::kan; PM5(pRK34), PM5 carrying plasmid-borne *bhuRSTUV*; PM5(pRK415), PM5 carrying plasmid vector control.

[b] Diameters of growth zones include the diameter of the well (6 mm). No growth occurred around wells containing the distilled-water diluent. —, no detectable growth stimulation.

suggesting that the Δ*bhuR*::kan chromosomal mutation may exert polar effects on the downstream *bhuSTUV* genes.

Growth stimulation bioassays were also used to determine whether *B. pertussis* could obtain iron from hemoglobins of various animal species. Wild-type strain UT25Sm1 showed similar levels of dose-dependent growth stimulation in response to bovine, porcine, rabbit, turkey, and human hemoglobins (data not shown). As predicted, Δ*bhuR*::kan mutant PM5 was incapable of utilizing any of the hemoglobins. Other bioassay experiments demonstrated that wild-type *B. pertussis* used both hemoglobin-haptoglobin and hemin-BSA complexes as iron sources, while the *bhuR* mutant strain was unable to utilize those complexes (data not shown). These data demonstrate that the *bhu* gene cluster is involved in the acquisition of iron from hemin and hemoglobin, as well as from haptoglobin and heme complexed with BSA. Furthermore, the data are consistent with the hypothesis that outer membrane receptor BhuR is capable of recognizing a range of heme compounds and of mediating transport of heme iron at levels sufficient to stimulate growth in an iron-limited medium.

Characterization of DNA sequences upstream of *bhuRSTUV*. The *B. pertussis bhu* contig identified in the Sanger Centre database did not include the genetic region encoding the putative BhuR start codon. To identify the 5′ limit of the *bhu* genetic system and to analyze potential upstream regulatory sequences, the nucleotide sequence of the *bhu* region absent from the database was determined using the cloned *B. pertussis* UT25 *bhu* DNA (Fig. 2). Analysis of this 871-bp *B. pertussis* UT25 nucleotide sequence and that of the overlapping *bhu* contig from the database predicted that the complete *bhuR* open reading frame encoded a precursor protein with a molecular mass of 92 kDa; upon cleavage of a predicted 21-amino-acid signal peptide, the mature BhuR protein would have a molecular mass of 90 kDa. An RPS-BLAST search for conserved protein domains predicted a TonB box C sequence (Pfam protein family [5] database domain: pfam 00593) in the carboxy-terminal region of BhuR. The FRAP and NPNL amino acid sequence motifs, which are highly conserved among hemin/hemoglobin receptors (67, 76), were also present in the corresponding regions of the deduced BhuR proteins of both *B. pertussis* and *B. bronchiseptica*, except that a tyrosine residue is substituted for phenylalanine in the FRAP motif and a serine replaces the second asparagine in the NPNL motif. A potential Fur-binding

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1    gcgcctggaccgcttcctgcgcgaactgagccgctaccgcccgggtacgctgcgctgcgaccccagggtggccgggctgcgtctgtccggcgtgttccag

101  ctggcccacaCCGACGACATCCTGCGCGCCCTGCCGGCGCTGCTGCCGGTGCAACTGTCCTATGTAACGCCGTACTGGATCACCGTCGGGCCCCGGCCCG

201  CCGGCGCCACGACGTGAAGAAGCGGCCAGCCATGAGCGCAGAACGTCCCTCGGCAAAAAAAATTCCTGTTCCCTGTCCGGTTTCGACGCGCCGGTGGTCA

301  TCGACAGCACAAGCACGCTCGTGCCTGCTCTCGATGGGAAAGGAAGATCATGGTTTACGCTTGCGATCAGGGCGCCCGCCGCGCCCGTCCCGCGCCGCCA
                                          -35                    -10

401  AGGCGCCCCCAAACGGCACTGGCCATGCGCGGCGCGCTGGCGGCATGCGCACTGGCCGGTACGCTGGCGGCCGCTCCCGCCGCCGCGCAGCCGACGGCGG
                              M  R  G  A  L  A  A  C  A  L  A  G  T  L  A  A  A  P  A  A  A  Q  P  T  A  A

501  CGCCCGCATCGGCGGGCGCGCGCGCCTGGCACATCGACGCCGGCCCCCTGGGCGAGGCCCTGGCGCGCTTTGCCGACCAGGCCGGCATTACCCTGCTGTA
       P  A  S  A  G  A  R  A  W  H  I  D  A  G  P  L  G  E  A  L  A  R  F  A  D  Q  A  G  I  T  L  L  Y

601  CGACCCCGCCGCGGTGCGCGGCCGCGCCAGCGCCGGCCTGCAAGGCGTGTACTCGGTGCCCGACGGCCTGGCGCGCCTGCTCGATGGCAGCGGCCTGGAC
       D  P  A  A  V  R  G  R  A  S  A  G  L  Q  G  V  Y  S  V  P  D  G  L  A  R  L  L  D  G  S  G  L  D

701  GCGCGCCAGCGCGGCGCCGGCACCTACGTGCTGCAGGCGCTGCCCGCCGGCCCGGTCGCCCAGCTGGCGCCGGTCACCATCGAGGCTgacggcgtgcgcg
       A  R  Q  R  G  A  G  T  Y  V  L  Q  A  L  P  A  G  P  V  A  Q  L  A  P  V  T  I  E  A  D  G  V  R  A

801  ccgatcccgcctgggcccgcaccgccacgcgccgcgagctcgacgcgcgccaggtgctcgactggagcgat...
       D  P  A  W  A  R  T  A  T  R  R  E  L  D  A  R  Q  V  L  D  W  S  D
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FIG. 2. *B. pertussis* nucleotide sequence encompassing the 5′ region of *bhuR*. An 871-bp region of the chromosome of strain UT25 was sequenced on both strands. The BhuR putative start codon is underlined, and the N-terminal 149-amino-acid region of BhuR is shown. The line above nucleotides 338 through 356 indicates the position of a potential Fur-binding site. Bracketed nucleotides represent the proposed ECF σ factor −10 and −35 promoter elements. Lowercase letters indicate nucleotides of the 871-bp region that overlap *B. pertussis* nucleotide sequence contigs found in the Sanger Centre database.

sequence identified 68 bases upstream of the BhuR gene start codon (Fig. 2) exhibited 58% identity with the 19-nt *E. coli* consensus Fur-binding sequence (19, 24). However, there was a G in place of the highly conserved T at nucleotide position 16 of the consensus Fur-binding sequence (71) and there was poor conservation of the characteristic inverted repeat, suggesting that this sequence may have limited Fur-binding activity.

Two contigs overlapping the 871-bp sequence (Fig. 2) were identified in the Sanger Centre *B. pertussis* genomic database. The original *bhu* contig exhibited 100% identity with the UT25 sequence over an 84-nt region, while a newly identified contig was 100% identical to the corresponding 110-nt end of the 871-bp sequence.

Identification of open reading frames encoding FecI and FecR homologs. Analysis of the two *B. pertussis* Sanger Centre contigs and the overlapping sequences obtained in this laboratory allowed examination of the *bhu* upstream region. A 5-kb DNA sequence was used in a BLASTX search at the NCBI which revealed two potential open reading frames (*orfI* and *orfR*) located 210 bp upstream of *bhuR* (Fig. 3). The OrfI and OrfR proteins showed the highest-scoring alignments with the FecI and FecR positive regulatory proteins, which control the ferric citrate uptake genes in *E. coli* (11, 75), and with *Pseudomonas putida* WCS358 PupI and PupR, which positively regulate the *pupB* ferric pseudobactin receptor gene (44) (Table 1). FecI and PupI function as alternative σ factors, and FecR and PupR are cytoplasmic membrane-bound regulatory proteins. An RPS-BLAST search using the predicted OrfI sequence revealed a domain highly characteristic of σ factors of the extracytoplasmic function (ECF) family (pfam domain: pfam 00776) (52).

No open reading frames that would be obvious targets of OrfIR regulation were identified upstream of *orfIR*, nor were any genes with apparent relevance to iron acquisition or *bhu* gene regulation found downstream of the *bhu* cluster. In the

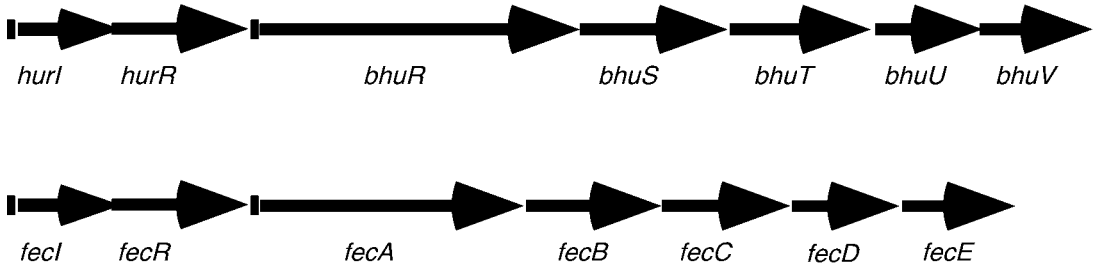

FIG. 3. Genetic organization of the *B. pertussis* heme iron uptake system and comparison with the *E. coli* ferric citrate uptake system. (Top) *B. pertussis* DNA region containing the *bhu* genes and the upstream *hurIR* open reading frames. Vertical bars upstream of *hurI* and *bhuR* show the positions of Fur-binding sequences which were predicted by nucleotide sequence analysis and which were assessed in vivo with the Fur repressor titration assay. (Bottom) Organization of the *fec* genetic system of *E. coli* (11), with putative Fur-binding sites denoted by vertical bars upstream of *fecI* and *fecA*. *fecI* and *fecR* encode positive regulatory proteins; *fecA* codes for the outer membrane ferric citrate receptor; *fecB*, *fecC*, *fecD*, and *fecE* encode periplasmic binding protein and cytoplasmic membrane transport components.

DNA sequence immediately upstream of *orfI*, two overlapping putative Fur-binding sequences exhibiting 64 and 74% identity to the consensus Fur-binding sequence were identified. A search for presumptive promoter region sequences revealed σ^{70}-like −10 and −35 regions (33) near the putative Fur boxes upstream of *orfI*, while putative ECF σ factor −10 and −35 regions (26, 52) were identified upstream of *bhuR* (Fig. 2). Due to the high degree of similarity among FecIR, PupIR, and the deduced *Bordetella* OrfIR proteins and the potential for OrfIR involvement in *bhu* gene regulation, we have tentatively designated these *B. pertussis* genes *hurIR,* for heme uptake regulators I and R.

Fur repressor titration analyses. The 2.4-kb *Xho*I-*Eco*RV DNA region 5′ to *bhuR* and the 0.8-kb *Eco*RI-*Xho*I DNA region upstream of *hurI* were each cloned to high-copy-number plasmid pGEM3Z and tested for functional Fur-binding activity by the Fur repressor titration assay employing *E. coli* indicator strain H1717 (71) (Fig. 4). In this in vivo assay, introduction of a functional Fur-binding site in multicopy relieves the repressive influence of Fur on the expression of the chromosomal *fhuF-lacZ* fusion under normally repressing high-iron growth conditions. These experiments demonstrated that the *bhuR* upstream region (p3Z82) had no apparent in vivo Fur-binding activity. This result is consistent with the low level of similarity of the *bhuR* upstream sequences to the consensus Fur-binding DNA sequence. In contrast, the *hurI* upstream region (p3Z88) exhibited a strong Fur-binding function, as evidenced by a high level of expression of *fhuF-lacZ* that was qualitatively equal to that conferred by positive control plasmid p3ZFBS, containing the consensus *E. coli* Fur-binding site (19, 24).

Analysis of heme-responsive protein expression. Multiple attempts to visualize the BhuR protein expressed in *B. pertussis* cells cultured under high- or low-iron conditions either in the presence or absence of hemin or hemoglobin were unsuccessful. In SDS-PAGE analyses, differences in stained or intrinsically radiolabeled proteins between wild-type UT25Sm1 and Δ*bhuR*::kan mutant PM5 could not be discerned. However, in parallel studies, *B. bronchiseptica bhuR* mutant BRM21 was constructed and was demonstrated in growth stimulation bioassays to be defective in heme iron acquisition (data not shown). Plasmid pRK34 carrying the wild-type *B. pertussis bhu* genes (Fig. 1) fully restored hemin and hemoglobin utilization to BRM21 (data not shown), indicating that the *bhu* system of *B. pertussis* can functionally complement the *bhu* mutation in *B. bronchiseptica*. Wild-type *B. bronchiseptica* strain B013N and *bhuR* mutant derivative BRM21 were cultured in SS medium under low-iron conditions with or without added hemin, and the total membranes were isolated and analyzed by SDS-PAGE. Wild-type cells grown in low-iron medium lacking hemin produced a ca. 90-kDa membrane protein, which was absent in the *bhuR* mutant membrane fraction (Fig. 5). This protein had an apparent molecular mass corresponding to the mass deduced for BhuR, and it was not produced by cells grown under iron-replete conditions (data not shown). Notably, production of the BhuR protein was elevated significantly in wild-type cells after 4 h of growth in the presence of hemin (data not shown) and was even more abundant after culture for 8 h in the presence of hemin (Fig. 5). The 90-kDa membrane protein was consistently absent in the *bhuR* mutant strain cultured under all growth conditions. This pattern of protein production by wild-type *B. bronchiseptica* cells suggests that iron-repressible expression of *bhuR* is responsive to the presence of hemin in the environment.

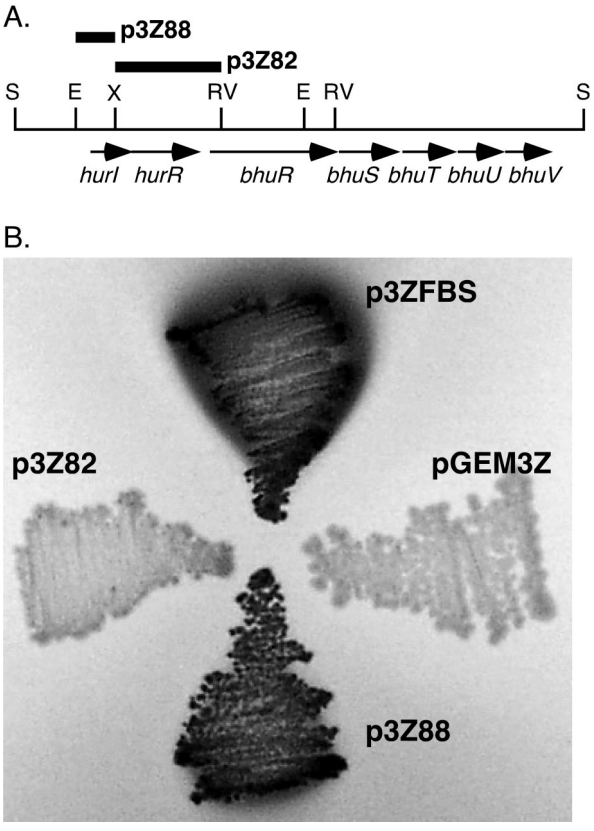

FIG. 4. Functional analysis of potential Fur-binding sites in the *hur-bhuR* DNA region. (A) Partial restriction map of the *hur-bhu* DNA region. Bars labeled p3Z88 and p3Z82, 0.8-kb *Eco*RI-*Xho*I fragment containing the *hurI* upstream region and 2.4-kb *Xho*I-*Eco*RV fragment containing the *bhuR* upstream region, respectively, which were cloned to high-copy-number vector pGEM3Z and tested for in vivo Fur binding. Abbreviations: E, *Eco*RI; RV, *Eco*RV; S, *Sal*I; X, *Xho*I. (B) Fur repressor titration assays were carried out as described in Materials and Methods using *E. coli* host strain H1717 plated on lactose MacConkey agar containing 40 μM iron. Dark areas of bacterial growth demonstrate β-galactosidase activity, which is indicative of functional Fur binding by the cloned DNA regions. Plasmids p3Z88 and p3Z82 are described in the legend for panel A; p3ZFBS contains the consensus *E. coli* Fur-binding sequence; pGEM3Z is the plasmid vector control.

DISCUSSION

When *B. pertussis* is starved for iron, it produces and utilizes its native siderophore alcaligin for iron acquisition (15, 53) and can also retrieve iron complexed with heterologous siderophores (6, 7), host-derived lactoferrin (61), transferrin (60, 61), hemoglobin (55), and hemin (1, 55). To date, the only *Bordetella* iron retrieval systems that have been characterized are those for ferric enterobactin transport (7) and for the biosynthesis and transport of alcaligin (8, 13, 32, 40, 41). In this study, we have identified a siderophore-independent iron ac-

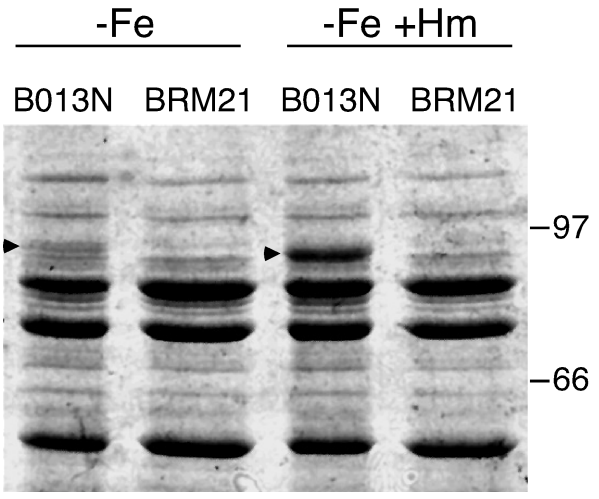

FIG. 5. Hemin-responsive BhuR expression in *B. bronchiseptica*. The growth of iron-starved *B. bronchiseptica* wild-type (B013N) and isogenic *bhuR* mutant (BRM21) cultures with or without added hemin is described in Materials and Methods. Total-membrane fractions were prepared from cells harvested 8 h after the addition of hemin and analyzed by SDS-PAGE. −Fe, cells grown under iron-depleted conditions; −Fe +Hm, cells grown under iron-depleted conditions and supplemented with 5 μM hemin. Arrowheads, positions of the putative BhuR outer membrane receptor protein. Migration positions of the protein standards and their molecular masses in kilodaltons are designated.

quisition system that is required by *Bordetella* spp. for the utilization of iron from heme compounds.

In the course of *B. pertussis* infection, virulence factors such as pertussis toxin (73), adenylate cyclase/hemolysin (21, 37), dermonecrotic toxin (49), and tracheal cytotoxin (22) are presumably elaborated, resulting in host cell dysfunction and damage to the mucosal epithelium (36). Extravasation of serum components, immune cells, and erythrocytes may ensue (59), and intracellular heme molecules may be liberated and subsequently serve as iron sources for *B. pertussis*. In this study, we identified the *B. pertussis bhu* genes, which encode functions required for utilization of iron from hemin and hemoglobin as well as other hemoproteins. Virtually identical *bhu* sequences were identified in the *B. bronchiseptica* genomic sequence database at the Sanger Centre. The *Bordetella bhu* genes are predicted to encode homologs of known prokaryotic heme utilization systems and are genetically organized in a cluster similar to those of other bacterial heme uptake systems. Based on amino acid sequence similarities with components of other heme utilization systems, BhuR is predicted to be the outer membrane receptor for hemin and hemoglobin. Other transport activities are hypothesized to be provided by the BhuT hemin-specific periplasmic binding protein, the BhuU cytoplasmic membrane permease protein, and BhuV, predicted to function as the ATPase required for heme transport across the cytoplasmic membrane. BhuS is similar to so-called hemin-degrading factors from *P. aeruginosa* (57), *Y. enterocolitica* (72), *Y. pestis* (74), and *S. dysenteriae* (51). Although a hemin-degrading activity for these proteins has not been demonstrated, Stojiljkovic and Hantke found that *hemS* was an essential gene in *Y. enterocolitica* and presented evidence that HemS expression prevented lethality in *E. coli* cells expressing the *Y. enterocolitica* HemR outer membrane receptor (72).

In our studies, wild-type *B. pertussis* was capable of acquiring iron from hemin, from hemoglobin from human, porcine, bovine, rabbit, and turkey sources, and from hemoglobin-haptoglobin and hemin-BSA complexes, while *bhuR* mutant PM5 was incapable of utilizing any of these compounds. These data indicate that the *bhu* genes are required for heme iron utilization in *B. pertussis* and that the BhuR outer membrane receptor is capable of recognizing a broad range of heme compounds. *Y. enterocolitica* receptor HemR also recognizes a variety of heme compounds including hemin, hemoglobin, myoglobin, hemopexin, and catalase and BSA- and human serum albumin-heme and haptoglobin-hemoglobin complexes (10). Though the mechanism of hemin and hemoglobin recognition by the outer membrane receptor and subsequent heme internalization remains unknown, Bracken and coworkers reported that a conserved histidine residue located between the FRAP and NPNL amino acid domains of HemR was important to the ability of *Y. enterocolitica* to effectively utilize hemin and heme-protein complexes (10). However, the *B. pertussis* and *B. bronchiseptica* BhuR proteins deduced from the available sequence data, as well as heme receptors PfhR (*Pseudomonas fluorescens*) and PhuR (*P. aeruginosa*) are predicted to lack the histidine residue in this region (67), suggesting that the mechanism of heme internalization by these receptors may be somewhat different from that of HemR.

In the present study, we were unable to visualize the BhuR protein by SDS-PAGE analysis of iron-starved or iron-replete wild-type *B. pertussis* grown in the presence or absence of hemin or hemoglobin. Because multiple proteins in the range of 80 to 95 kDa are expressed under iron starvation conditions (12), it is possible that *B. pertussis* BhuR comigrates in electrophoretic gels with one or more other proteins. However, comparative analysis of wild-type *B. bronchiseptica* and its *bhuR* mutant derivative revealed a membrane protein corresponding to BhuR in iron-starved wild-type cells that was absent in the *bhuR* mutant grown under the same low-iron conditions. Remarkably, iron-starved wild-type *B. bronchiseptica* cultures supplemented with hemin demonstrated dramatically enhanced production of BhuR, suggesting that *bhuR* expression is responsive to the presence of heme.

Expression of the *Vibrio cholerae* (56), *Y. pestis* (74), *Y. enterocolitica* (72), and *P. aeruginosa* (57) heme utilization genes is negatively regulated by iron through the Fur repressor. A potential *B. pertussis* Fur-binding site identified upstream of *bhuR* exhibited essentially no in vivo Fur-binding activity, suggesting that regulation of the *bhu* system differs from that of other known microbial heme systems. Transcription of *bhuR* may be iron repressed indirectly, perhaps through Fur repression of putative positive regulatory gene *hurI*, or may be unresponsive to iron concentration. However, because *B. bronchiseptica* produces BhuR only under iron-restricted growth conditions, the latter possibility seems unlikely. In addition to repression by Fur, positive transcriptional regulation has been demonstrated for some siderophore system genes (23), including the *Bordetella* alcaligin genes (8, 16) and those for ferric citrate uptake in *E. coli* (11). This positive regulation occurs only after Fur derepression and in the presence of the cognate iron compound. To date, no gram-negative bacterial heme iron

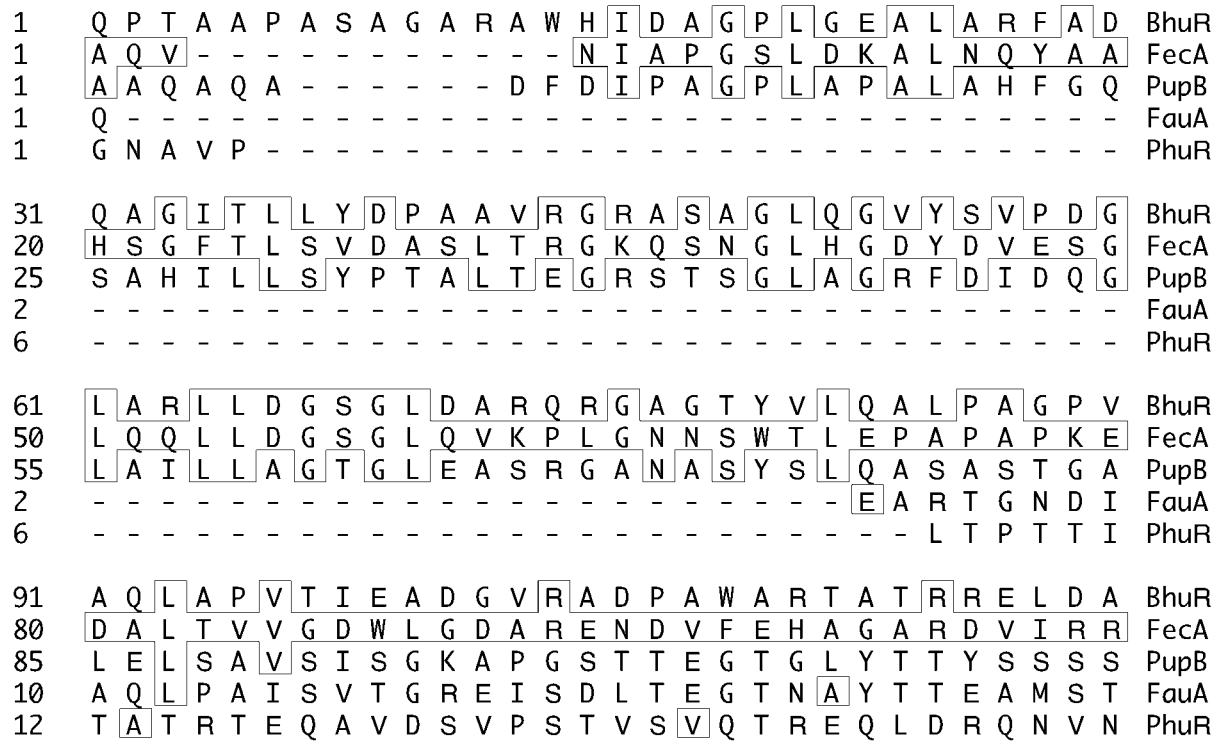

FIG. 6. Amino acid sequence alignments of BhuR with selected bacterial heme and ferric siderophore receptors. The primary amino acid sequences of the deduced mature proteins were aligned using the CLUSTAL program. Shown is the alignment of the N-terminal regions of the proteins; significant similarity between the extended N-terminal domains of BhuR, FecA and PupB can be seen. Amino acid residues in boxes are those that match the FecA amino acid sequence. Proteins: BhuR, *B. pertussis* heme receptor; FecA (GenBank accession no. AAC77247), *E. coli* ferric citrate receptor; PupB (P38047), *P. putida* WCS358 ferric pseudobactin receptor; FauA (AAD26430), *B. pertussis* ferric alcaligin receptor; PhuR (AF055999), *P. aeruginosa* heme receptor.

acquisition system has been reported to require positive transcriptional regulation. In gram-positive pathogen *Corynebacterium diphtheriae*, the genes encoding the heme transport apparatus are expressed constitutively (25) whereas transcription of *hmuO*, encoding a heme oxygenase, is activated by a two-component regulatory system which responds to the presence of heme or hemoglobin in the environment (63). In our study, the *hurIR* open reading frames identified upstream of the *B. pertussis bhu* gene cluster are predicted to encode homologs of the *E. coli* FecI ECF σ factor/FecR family of regulatory proteins, suggesting that the *bhu* system may be positively regulated in a manner similar to that for the *fec* system. Stiefel and coworkers recently identified FecR homologs from a variety of bacterial species by genomic database BLAST searches (70). In that study, the authors identified the DNA sequence contigs in the Sanger Centre genomic database predicted to encode the HurR proteins of both *B. pertussis* and *B. bronchiseptica*. The HurR proteins of both species share the three conserved tryptophan residues characteristic for the FecR class of transmembrane regulatory proteins (70).

Several lines of evidence suggest that *hurIR* may be involved in positive transcriptional regulation of the *B. pertussis bhu* genes. First, no other open reading frames apparently relevant to iron acquisition or iron-regulated gene expression were identified near *hurIR* or the *bhu* genes. Second, the *E. coli fecIR* genes are directly adjacent to the *fec* genes encoding the ferric citrate transport machinery in a pattern strikingly similar to that of the *hurIRbhuRSTUV* genes (Fig. 3). Third, upstream putative *bhuR* promoter sequences are predicted to require an ECF σ factor for transcription. The *hurI* gene encodes a putative ECF σ factor, and upstream of *hurI* is a DNA sequence that exhibited strong Fur-binding activity, consistent with a role for HurI in iron metabolism. Similarly, *fecI* is iron regulated via Fur and the *fecA* ferric citrate receptor gene has an ECF σ factor-dependent promoter. The HurR protein is highly similar to the FecR and PupR cytoplasmic membrane proteins and contains one predicted membrane-spanning region. The conserved C-terminal regions of FecR and PupR are also highly conserved in HurR; the C-terminal one-third of HurR was 41 and 43% similar to the same regions of PupR and FecR, respectively. This C-terminal region of FecR interacts with the periplasmic N-terminal extension of the FecA outer membrane ferric citrate receptor to effect positive regulation of *fec* genes (27, 43). Most importantly, analysis of the BhuR outer membrane receptor amino acid sequence revealed the presence of this highly conserved extended N-terminal region (Fig. 6). Thus, BhuR appears to be a member of this family of outer membrane iron receptors that function in systems positively controlled by a FecIR-type transcriptional regulatory system. Amino acid sequence comparisons with the *P. aeruginosa* PhuR heme receptor and the *B. pertussis* FauA ferric alcaligin receptor revealed that these proteins lack the N-terminal extension characteristic of the FecA, PupB, and BhuR receptors (Fig. 6). The presence of this highly conserved N-terminal

extension in BhuR is consistent with the notion that expression of the *B. pertussis bhu* genes may be positively regulated by an alternative σ factor-dependent system encoded by *hurIR*. Finally, the observation that iron-stressed *B. bronchiseptica* cells dramatically enhance the production of BhuR in response to hemin further suggests the involvement of a positive regulatory system controlling receptor gene expression. Experiments aimed at defining the potential role of *hurIR* in *bhu* gene regulation are in progress.

The specific iron sources upon which *B. pertussis* relies for in vivo growth are unknown. It is clear, however, that this organism possesses genes encoding multiple iron acquisition systems. During the course of infection, from the inhalation of bacteria in microaerosols to colonization and host tissue injury, it may be expected that the genes encoding all of these iron acquisition systems are first expressed after Fur derepression. These genetic systems may then be individually positively controlled primarily by the availability of the cognate iron source by a priority regulation mechanism typified by the transcriptional control of the native siderophore system by the AlcR regulator with alcaligin as the inducer (8, 16). In the latter stages of the infectious process, when there is considerable host cell damage, intracellular heme compounds may be released, potentially providing a priority activation signal for enhanced transcription of the *Bordetella bhu* genes.

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