

# RhuR, an Extracytoplasmic Function Sigma Factor Activator, Is Essential for Heme-Dependent Expression of the Outer Membrane Heme and Hemoprotein Receptor of *Bordetella avium*

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**Genes involved in iron (Fe) acquisition often are regulated in response to the local availability of Fe. In many bacteria, Fe-dependent responsiveness is mediated by Fur, a global Fe-dependent transcriptional repressor. Tighter regulatory control of Fur-responsive genes is afforded by incorporating additional regulators into Fur-dependent regulatory cascades. RhuI, a Fur-dependent extracytoplasmic function sigma factor of *Bordetella avium*, in response to the dual stimulation of Fe starvation and the presence of heme (or hemoproteins), regulates  $P_{bhuR}$ , a heme-responsive promoter which directs expression of the *bhuRSTUV* heme utilization operon. While BhuR, the outer membrane heme receptor, and RhuI have been shown to be indispensable for heme-dependent activation of  $P_{bhuR}$ , collateral components of the regulatory cascade have not been described. In this investigation, RhuR, an integral cytoplasmic membrane protein with homology to anti-sigma factors, is shown to be an essential activator of  $P_{bhuR}$  expression. The functional domain of RhuR required for heme-dependent activation of  $P_{bhuR}$  expression was mapped to the N-terminal 97 amino acids of the protein by use of a chimeric RhuR-BlaM fusion. Expression of the chimera in a *rhuR* mutant rendered  $P_{bhuR}$  constitutive, thereby decoupling the promoter from heme dependency. Growth studies confirmed that *B. avium* requires RhuR for optimal utilization of hemoglobin, but not hemin, as a sole source of nutrient Fe. These data imply that *B. avium* expresses, in addition to the BhuR heme/hemoprotein utilization system, an alternative RhuR-independent heme utilization mechanism. A model is proposed in which RhuR is the functional bridge between BhuR and RhuI in a heme-dependent regulatory cascade.**

Iron (Fe), an essential element for nearly all pathogenic bacteria, must be derived from the host. In hosts, however, free Fe is usually sequestered by high-affinity molecules such as hemopexin, haptoglobin, and ferritin. Fe is also found coordinately bound with molecules such as hemoglobin, myoglobin, and transferrin. Efficient scavenging of Fe and Fe-containing molecules by the sequestering complexes reduces the concentration of the metal in the tissues and fluids of vertebrates well below the critical concentrations required for bacterial growth, rendering these compartments as inhospitable environments for colonization. To counteract this blockade to successful colonization, bacteria have evolved sophisticated Fe acquisition systems for removing the metal from the diverse sequestering molecules (4). Bacteria express a variety of Fe acquisition systems, each specific for one or more of the various host-specific Fe-containing molecules (4).

Expression of genes encoding the various Fe acquisition systems is rigidly regulated in response to environmental Fe availability and to the intracellular Fe concentration. When Fe is abundant, expression of the uptake systems is repressed. The capacity to downregulate expression of these systems when unneeded is an adaptive response that conserves metabolic resources. Tight control of Fe uptake also evolved to prevent accumulation of lethal concentrations of the metal in the bac-

terial cytoplasm. Bacteria have a variety of intracellular Fe sequestering systems for detoxification of the metal. Yet, if these systems are overwhelmed by uncontrolled Fe uptake, the result can be lethal. Fe is a potent oxidizer and has the capacity to damage DNA and a variety of other indispensable biomolecules. Extensive conservation of certain Fe-dependent regulators across genera suggests that subtle yet rigid control of expression of genes encoding Fe acquisition systems is crucial to most bacteria. For example, Fur (Fe uptake regulator), a global Fe-dependent transcriptional repressor which controls a variety of Fe acquisition systems, is highly conserved in virtually all bacteria. The capacity of Fur to respond to Fe stress is derived from the molecule's innate ability to reversibly bind Fe. When intracellular Fe is abundant, Fur is complexed with Fe. Binding of Fe stimulates binding of the protein to a 19-bp nucleotide sequence that is located proximal to Fur-dependent promoters. Binding of Fur to the "Fur box" blocks transcription of the Fur-regulated cistron (or polycistron). Upon Fe starvation, the metal dissociates from Fur, which decreases the repressor's affinity for the Fur box. Release of Fur from the Fur box derepresses transcription of the regulated gene (7).

In many cases, Fur is sufficient to adequately control expression of a specific gene. In other cases, genes encoding a particular Fur-dependent Fe acquisition system may require an additional level of regulation. These more complicated regulatory systems often operate in concert with Fur to activate specific genes under more limited environmental circumstances. Secondary regulatory systems have been described in several bacteria: siderophore expression in *Pseudomonas*

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*aeruginosa* is controlled by PchR, an AraC-type regulator which responds to extracellular pyochelin (12); genes for heme biosynthesis in *Bradyrhizobium japonicum* are controlled by Irr in response to internal Fe and heme stores (11); and *irgA* synthesis in *Vibrio cholerae* is induced by the LysR-type regulator IrgB (42). Recently, it was demonstrated that extracytoplasmic function (ECF) sigma factors are essential ancillary components in some Fur-dependent regulatory cascades (4).

The ECF class of  $\sigma^{70}$ -type sigma factors (24) are small, functionally modifiable proteins that provide promoter specificity to core RNA polymerase (RNAP) (44). ECF sigma factors have been found to regulate a wide variety of cellular activities that mediate adaptive responses of bacteria to the local environment. These adaptive responses include the regulation of motility and carotenogenesis in *Myxococcus xanthus* (10, 41), toxin production in *Clostridium difficile* (26), and mycelium formation in *Streptomyces coelicolor* (3). Unlike  $\sigma^{70}$ , the housekeeping sigma factor, the transcriptional activities of ECF sigma factors are modulated as a result of signal transduction cascades activated by an extracytoplasmic inducer molecule (24). Evidence is accumulating that bacterial pathogens have evolutionarily recruited ECF sigma factors for regulation of various virulence molecules (20, 45). Fur-dependent ECF sigma factors have been described which control genes for siderophore biosynthesis (22, 33, 40) and genes encoding proteins essential for uptake of exogenous Fe complexes (18, 19, 38, 39).

Interactions of ECF sigma factors with their affiliated regulators may be simple, complicated, or absent. While expression of the siderophore biosynthesis regulators PfrI of *Pseudomonas putida* and PbrA of *Pseudomonas fluorescens* is Fur dependent, no other regulatory partners for these ECF sigma factors have been identified (33, 40). In contrast, the ECF sigma factors FecI of *Escherichia coli* (39), PupI of *P. putida* (19), PvdS of *P. aeruginosa* (21), and HurI of *Bordetella pertussis* (38), which are associated with regulation of systems for uptake of various Fe sources, are dependent upon secondary transmembrane regulators for their functions. A useful paradigm to demonstrate the interdependence of the ECF sigma factor and its cognate transmembrane regulator is the FecI/FecR-regulated ferric dicitrate acquisition system of *E. coli*. Conditional transcription of the *fec*-encoded genes for ferric dicitrate transport is controlled by FecI, a modulated Fur-dependent sigma factor (2). Binding of ferric dicitrate to FecA, the outer membrane ferric dicitrate receptor, stimulates FecR, an integral cytoplasmic membrane protein (17) which transduces a signal to FecI, a cytoplasmic ECF sigma factor. FecI, in association with RNAP, upregulates transcription of *fecABCDE* (6, 25, 30). Although FecR is necessary to activate FecI, the precise molecular mechanism by which the dicitrate-dependent signal is transduced from FecR to FecI has yet to be elucidated. A similar ECF-based regulatory system for pseudobactin-dependent induction of PupI (ECF sigma factor) by PupR (cytoplasmic membrane PupI regulator) has also been described in *P. putida* (19).

*Bordetella avium*, a gram-negative coccobacillus, is the causative agent of avian coryza (bordetellosis). Since the pathology of coryza exhibits a strong similarity to whooping cough, an upper respiratory disease caused by the obligate human pathogen *B. pertussis*, *B. avium* has been employed as a surrogate

pathogen to study *B. pertussis* pathogenesis (28). Previously, our laboratory described a heme and hemoprotein acquisition system (*bhuRSTUV*) in *B. avium* that is regulated by RhuI, a Fur-dependent ECF sigma factor, in concert with BhuR, the outer membrane heme receptor (18, 28). A similar locus (*hurIRbhuRSTUV*) was recently described in *B. pertussis* and *Bordetella bronchiseptica* (37). Using *B. bronchiseptica* as a model system, Vanderpool and Armstrong (38) showed that the *B. pertussis* *bhuR* and *hurI* genes are required for heme-dependent induction of *bhuR* expression (38). In this study, we describe the functional role of RhuR, a third component of the heme-dependent regulatory cascade, in the regulation of heme acquisition by *B. avium*. Database searches revealed that RhuR shares homology with anti-sigma factors FecR of *E. coli* (30) and PupR of *P. putida* (19), which are intimately involved in the regulatory activities of the Fe-dependent ECF sigma factors FecI and PupI, respectively. Based on these findings, it was postulated that RhuR functionally bridges the regulatory cascade from BhuR to RhuI (18). In this report, cell fractionation studies mapped the protein to the cytoplasmic membrane. In addition, experiments using fusion proteins revealed that the heme-dependent RhuI activation domain of RhuR is located within the N-terminal portion of this membrane protein. The critical significance of RhuR in the efficient utilization of hemoglobin, but not of hemin, as a source of nutrient Fe was also established.

#### MATERIALS AND METHODS

**Strains, media, reagents, and plasmids.** Bacterial strains and plasmids used in this study are listed in Table 1. *B. avium* strains were maintained on brain heart infusion (BHI) agar or broth (Difco Laboratories, Detroit, Mich.). *E. coli* strains were cultured on Luria-Bertani agar. BHI was supplemented with 150  $\mu$ M FeSO<sub>4</sub> for Fe-replete growth conditions. Fe-stressed growth conditions were established by addition of ethylenediamine di-*o*-hydroxy-phenylacetic acid (EDDHA; Sigma Biochemicals, St. Louis, Mo.) to BHI at a final concentration of 100  $\mu$ M. EDDHA at a concentration of 300 to 400  $\mu$ M was used to inhibit growth of the bacterium in the absence of hemin or other hemoproteins. Unless otherwise noted, ampicillin was used at a concentration of 200  $\mu$ g/ml, rifampin was used at 10  $\mu$ g/ml, streptomycin was used at 100  $\mu$ g/ml, tetracycline was used at 10  $\mu$ g/ml, and gentamicin was used at 10  $\mu$ g/ml. Antibiotics were obtained from Sigma Biochemicals and Amresco (Solon, Ohio). Biochemical reagents were purchased from Sigma Biochemicals. Restriction enzymes, DNA-modifying enzymes, and *Taq* polymerase were obtained from Fermentas, Inc. (Hanover, Md.) and Invitrogen (Carlsbad, Calif.). Deionized water with an electrical resistance of >18 M $\Omega$  was used for all solutions.

**Construction of *rhuR* deletion strain.** *rhuR* was deleted from the *B. avium* 4169rif chromosome by use of allelic exchange. The mutant gene used in allelic exchange was engineered by PCR splicing by overlap extension (SOEing). Sequences flanking *rhuR* in the 4169rif chromosome were amplified by PCR using primers designed to amplify the 631 bp located immediately 5' of the ATG start codon of *rhuR* ( $\Delta$ *rhuR*-a, 5'-TCCGAGCTCTGACCTCGCCTGAGCCT-3' [*Sac*I site underlined]);  $\Delta$ *rhuR*-b, 5'-CTATTTAGTAACAGGAATCATTATCATGACGCATCCATCAC-3') and the 451 bp immediately 3' of the TGA stop codon ( $\Delta$ *rhuR*-c, 5'-TAAATGATTCCTGTACTAAATAGATT-3';  $\Delta$ *rhuR*-d, 5'-GCTCTAGACACATAGGTTGTGCC-3' [*Xba*I site underlined]).  $\Delta$ *rhuR*-b was synthesized with a 5' extension (indicated in bold) complementary to  $\Delta$ *rhuR*-c, which enabled the two PCR products to anneal. Primary amplicons were produced using *Taq* polymerase (1 to 10 U) from the template pAD3 under the following conditions: 1 $\times$  PCR buffer (Promega, Madison, Wis.), 10% dimethyl sulfoxide, 150  $\mu$ M deoxynucleoside triphosphates (dNTPs), 1  $\mu$ M concentration of each oligonucleotide primer; 94°C for 1 min, 45°C for 1 min, and 72°C for 1 min; 25 cycles. PCR products were purified using the GFX purification kit (Amersham Pharmacia Biotech, Inc., Piscataway, N.J.). The SOEing reaction was performed as follows: 1 $\times$  PCR buffer, 10% dimethyl sulfoxide, 250  $\mu$ M dNTPs, 1  $\mu$ M  $\Delta$ *rhuR*-a, 1  $\mu$ M  $\Delta$ *rhuR*-d, 2  $\mu$ l of each primary product (10 to 50 ng); *Taq* polymerase (1 to 10 U); 94°C for 1 min, 45°C for 1 min, and 72°C for

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source
<b>B. avium strains</b>		
4169rif	wt strain; Stp <sup>r</sup> Rif <sup>r</sup>	28
4169rifΔ <i>rhuR</i>	Chromosomal <i>rhuR</i> deletion of 4169rif; Stp <sup>r</sup> Rif <sup>r</sup>	This study
4169rif <i>bhuR::kan</i>	Insertion of Kan <sup>r</sup> cassette into <i>bhuR</i> of 4169rif; Stp <sup>r</sup> Rif <sup>r</sup>	28
<b>E. coli strains</b>		
DH5αF <sup>'</sup> tet	φ80 <i>dlacZM15</i> Δ( <i>lacZYA-argF</i> )U169 <i>deoR recA1 phoA hsdR17</i> (r <sub>K</sub> <sup>-</sup> m <sub>K</sub> <sup>+</sup> ) <i>supE44λ<sup>-</sup> thi-1 gyrA96 relA1</i> [F <sup>'</sup> <i>proA B lac<sup>q</sup>ZΔM15 Tn10</i> (Tet <sup>r</sup> )]	Invitrogen
HB101	F <sup>-</sup> Δ( <i>gpt-proA</i> )62 <i>leuB6 glnV44 ara-14 galK2 lacY1</i> Δ( <i>mcr-mrr</i> ) <i>rpsL20 xyl-5 mtl-1 recA13</i> ; Stp <sup>r</sup>	New England Biolabs
MC4100λ <i>pir</i>	F <sup>-</sup> <i>araD139</i> Δ( <i>argF-lac</i> )U169 <i>rpsL150 relA1 ffbB5301 deoC1 ptsF25 rbsR</i> (λ <i>pir</i> )	34
<b>Plasmids</b>		
pBluescript KS(+)	Cloning vector; Amp <sup>r</sup>	Stratagene
pET21a	Protein expression vector; P <sub>T7</sub> ; Amp <sup>r</sup>	Novagen
pMECA	Cloning vector; Amp <sup>r</sup>	36
pCVD442tet	Allelic exchange vector; λ <i>pir</i> dependent; <i>sacB</i> ; Amp <sup>r</sup>	28
pRK415	Conjugative expression shuttle vector for <i>B. avium</i> and <i>E. coli</i> ; P <sub>lac</sub> ; Tet <sup>r</sup>	16
pRK2013	Conjugative helper plasmid; Kan <sup>r</sup>	8
pAEK26	<i>rhuR</i> ligated into pRK415 at the <i>Xba</i> I/ <i>Eco</i> RI sites	This study
pAEK26.3	<i>rhuR</i> -His <sub>6</sub> ligated into pRK415 at the <i>Xba</i> I/ <i>Eco</i> RI sites	This study
pAEK26.1	<i>rhuR</i> ligated into pET21a at the <i>Nde</i> I/ <i>Eco</i> RI sites	This study
pAEK21	5' 291 bp of <i>rhuR</i> fused to <i>blaM</i> ligated into pRK415 at the <i>Bam</i> HI/ <i>Hind</i> III sites	This study
pAEK23	291 bp of 5' end of <i>rhuR</i> ligated into pRK415 at the <i>Bam</i> HI/ <i>Eco</i> RI sites	This study
pAD3	pBluescript KS(+) encoding the 5' terminus of <i>bhuR</i> and 7.4 kbp of upstream sequence	28
pERM1	3-kbp <i>Eco</i> RI fragment encoding <i>rhuIR</i> -P <sub><i>bhuR</i></sub> - <i>bhuR</i> , in pMECA	28
pAEK16	<i>rhuR</i> deletion allelic exchange clone in pCVD442tet	This study
pAEK20.1	Mutated <i>rhuR</i> allele (G569A) in pET21a	This study
pAEK25	<i>rhuIR</i> locus in pBluescript KS(+) at the <i>Eco</i> RI/ <i>Bam</i> HI sites	This study
pDJM41	P <sub><i>bhuR</i></sub> - <i>lacZYA</i> reporter; Amp <sup>r</sup> Gen <sup>r</sup>	18

1 min; 25 cycles. The 1,086-bp product was ligated into the allelic exchange vector pCVD442tet at the *Xba*I and *Sac*I sites to produce pAEK16, which was conjugated into *B. avium* 4169rif. Transconjugants were selected on BHI agar containing tetracycline. To select for the secondary recombination event, transconjugants were plated on BHI agar containing 20% sucrose. Sucrose-resistant clones were screened by colony lift hybridization, Southern hybridization, and colony PCR to assure that *rhuR* had been deleted without inadvertent disruption of nucleotide sequences proximal to *rhuR* in the *rhu-bhu* locus.

**Cloning wt *rhuR*.** The *rhuR* open reading frame (ORF) was amplified by PCR using primers *rhuR*-5N-2 (5'-GGAATTCATATGAGCGCAGCCGCGGT-3' [*Nde*I site underlined]) and *rhuR*-3E (5'-TCTATTTAGTAACAGGAATTCAT TTA-3' [*Eco*RI site underlined]). The PCR conditions using pERM1 as a template were as follows: 1× ThermalACE buffer, 1× dNTP mix, 240 nM each primer, 4 U of ThermalAce polymerase; 95°C for 30 s, 45°C for 30 s, and 72°C for 1.25 min; 30 cycles. The 958-bp amplification DNA fragment was ligated into pET21a at the *Nde*I and *Eco*RI sites to produce pAEK20.1, in which the ORF was oriented appropriately downstream of a vector-encoded ribosomal binding site. Sequencing revealed the presence of a PCR-derived nucleotide mutation in the *rhuR* coding sequence. To engineer pAEK26.1, a plasmid encoding a wild-type (wt) *rhuR*, pAEK20.1, was digested with *Cla*I and *Nco*I and the 657-bp fragment containing the point mutation was replaced with the corresponding *Cla*I/*Nco*I fragment from pAEK25. A 1,002-bp DNA fragment obtained by digestion of pAEK26.1 with *Eco*RI and *Xba*I was ligated into the equivalent restriction sites of the conjugal shuttle vector pRK415. The ligation properly oriented the *rhuR* gene proximal to the *lac* promoter for isopropyl-β-D-thiogalactopyranoside-inducible expression in *E. coli* and constitutive expression in *B. avium*. This shuttle plasmid was designated pAEK26.

**β-Galactosidase assay.** Expression of the *lacZYA* reporter gene in pDJM41 was determined by measuring β-galactosidase activity (18). Briefly, overnight cultures of Fe-stressed cells were used to inoculate secondary cultures in desired culture media. Secondary cultures were incubated at 37°C for 16 to 20 h. Bacteria in 1.0 ml of culture were pelleted by centrifugation for 5 min at 5,000 × g. Cells were resuspended in Z buffer (60 mM Na<sub>2</sub>HPO<sub>4</sub> · H<sub>2</sub>O, 40 mM NaH<sub>2</sub>PO<sub>4</sub> · H<sub>2</sub>O, 10 mM KCl, 1 mM MgSO<sub>4</sub> · H<sub>2</sub>O, 38 mM β-mercaptoethanol) and the optical density at 600 nm (OD<sub>600</sub>) of the cell suspension was adjusted to 0.28 to 0.70. After diluting 400 μl of the suspension with 400 μl of Z buffer, cells were permeabilized by addition of 45 μl of 0.1% sodium dodecyl sulfate (SDS) and 90

μl of chloroform. Cell suspensions were vortexed for 10 s, followed by a 15-min incubation at 30°C. Enzymatic reactions were initiated by addition of 160 μl of a 4-mg/ml solution of *o*-nitrophenyl-β-D-galactopyranoside. Following incubation at 30°C, samples were observed for development of a yellow color, at which point 400 μl of 1 M Na<sub>2</sub>CO<sub>3</sub> was added to terminate the reaction. After a brief centrifugation to pellet debris and chloroform, the OD<sub>420</sub> and OD<sub>550</sub> of each reaction were recorded and the relative β-galactosidase activity was calculated by use of the following formula (27):  $\{1,000 \cdot [\text{OD}_{420} - 1.75(\text{OD}_{550})]\} / (t)(0.4) (\text{OD}_{600})$ , with *t* = time of reaction in minutes.

Relative enzymatic activities are reported as the mean of triplicate assays and were derived from at least two independent experiments.

**Endpoint growth assay.** All strains were inoculated into BHI broth containing 100 μM EDDHA and appropriate antibiotics. After overnight incubation at 37°C, these primary Fe-starved cultures were used to inoculate triplicate 3-ml BHI broth cultures under the particular growth conditions to be investigated. All cultures were supplemented with 400 μM EDDHA, a concentration at which growth of *B. avium* is inhibited (28). An Fe-replete environment was produced by supplementing the culture with 300 μM FeSO<sub>4</sub>. Hemin or turkey hemoglobin was added to the cultures at desired concentrations. Endpoint culture densities after 24 h of incubation at 37°C were ascertained by use of a Beckman DU640 spectrophotometer (Fullerton, Calif.) using the average culture density obtained from three independent cultures under a given culture condition.

**Cell fractionation.** For cell fractionation experiments, 5 ml of bacteria obtained from overnight Fe-limited BHI broth (BHI plus 100 μM EDDHA) cultures were used to inoculate 500 ml of Fe-replete (BHI plus 150 μM FeSO<sub>4</sub>), Fe-stressed (BHI plus 100 μM EDDHA), or heme-induced (BHI plus 100 μM EDDHA plus 1 μM hemin) broth. After overnight incubation at 37°C, cells from 1.0 ml of culture were pelleted by centrifugation and resuspended in 1× SDS-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. These samples represented the whole cell fraction. Total membrane, outer membrane, and cytoplasmic membrane (sarcosine extract) fractions were prepared from the remaining culture by the differential centrifugation and lauroyl sarcosine extraction method of Leyh and Griffith (23). Final membrane fractions were resuspended in sterile deionized H<sub>2</sub>O and stored at -70°C. The total protein concentration of each fraction was obtained using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, Calif.) and a bovine serum albumin standard. Solubilized proteins were separated by SDS-PAGE using 12% polyacrylamide

gels. To visualize resolved proteins in the cell fractions, gels were stained with colloidal Coomassie brilliant blue (29). For Western immunoblotting for RhuR-His<sub>6</sub> polypeptides, cell fractions were electrotransferred to BioTraceNT nitrocellulose filters (Pall Corporation, Ann Arbor, Mich.), which were subsequently probed with INDIA HisProbe-horseradish peroxidase (HRP) (Pierce, Rockford, Ill.) using the manufacturer's protocol. Rabbit polyclonal anti-β-lactamase antibodies (5 Prime-3 Prime, Inc., Boulder, Colo.) and peroxidase-conjugated anti-rabbit immunoglobulin G (whole molecule; Sigma Biochemicals) antibodies were used to detect RhuR-BlaM fusion protein. INDIA-HisProbe-reactive and anti-BlaM-immunoreactive proteins on the filters were visualized using the Renaissance Western blot chemiluminescence reagent (DuPont NEN Research Products, Boston, Mass.) and blue-sensitive autoradiographic film (Marsh Bio-products, Rochester, N.Y.).

**Nucleotide sequencing and analysis.** Nucleotide sequencing was performed by the Biopolymer Facility at Roswell Park Cancer Institute (Buffalo, N.Y.). *B. pertussis*, *Bordetella parapertussis*, and *B. bronchiseptica* sequences were obtained from the Sanger Center database (*Bordetella* Sequencing Group, Sanger Center; <http://www.sanger.ac.uk/Projects>). Sequence analysis was performed using the Wisconsin Package version 9.0 software package (Genetics Computer Group, Madison, Wis.) and ClustalW (<http://www.ebi.ac.uk/clustalw>).

## RESULTS

**RhuR has homology to anti-sigma factors.** RhuI, an ECF sigma factor located in the cytoplasm of *B. avium* (GenBank accession number AY095952) has been shown to induce  $P_{bhuR}$ , a heme-responsive promoter controlling expression of BhuR, the outer membrane heme receptor of the bacterium (18). Subsequently, we found that heme-dependent induction of  $P_{bhuR}$  expression was absent in a  $\Delta rhuI$  mutant strain of *B. avium* (data not shown). Heme-dependent induction of  $P_{bhuR}$  was restored when a plasmid copy of *rhuI* was introduced into the mutant. These results provided evidence that heme-dependent induction of  $P_{bhuR}$  in *B. avium* requires expression of RhuI. Since BhuR and RhuI are separated by the plasma membrane, a third component to functionally bridge these two polypeptides in the regulatory cascade was hypothesized. As genes encoding accessory regulatory components are often genetically linked, it was predicted that this bridging protein would most likely be encoded by a gene located proximal to *bhuR* and *rhuI* in the *B. avium* chromosome (18). Nucleotide sequencing of the region of the *B. avium* chromosome between the 3' end of *rhuI* and the 5' end of *bhuR* revealed a 921-bp ORF with a coding capacity for a polypeptide of 307 amino acids with a predicted molecular mass of 32.8 kDa (18). A 29-amino-acid sequence located at the N terminus of this predicted polypeptide containing an Ala-X-Ala motif (amino acid positions 27 to 29) was consistent in structure with a typical signal peptide commonly observed in proteins destined for *sec*-dependent transport to the cytoplasmic membrane. Signal peptidase-dependent cleavage of this putative signal peptide would produce a mature protein of 29.7 kDa. From one to three putative transmembrane domains were predicted by computer analysis of the polypeptide sequence. Collectively, these characteristics were consistent with those expected for a cytoplasmic membrane-bound transducer that would functionally link BhuR and RhuI. For the sake of consistency, this ORF was designated *rhuR*.

Protein similarity searches of the database revealed that RhuR has homology to a family of proteins which regulate the activity of ECF sigma factors (Fig. 1), including the HurR proteins of *B. bronchiseptica* and *B. pertussis* (39% identity and 51% similarity). RhuR also exhibited significant homology to

PupR (31% identity and 46% similarity), a predicted cytoplasmic membrane protein which is required for proper regulation of PupI, the pseudobactin-dependent ECF sigma factor of *P. putida* (19). FecR, which regulates the activity of FecI, the ECF sigma factor of *E. coli* essential for uptake of ferric dicitrate, exhibits slightly less similarity to RhuR (28% identity and 42% similarity) (30). Three Trp residues (Trp18, Trp38, and Trp49) located near the N terminus of RhuR are conserved in HurR of *B. pertussis*, HurR of *B. bronchiseptica*, PupR, and FecR (Fig. 1). In FecR, these tryptophan residues have been shown to be required for ferric dicitrate-dependent induction of the *fecABCDE*-encoded ferric dicitrate acquisition system (35). Experiments to determine the importance of the three tryptophan residues in RhuR in heme-dependent regulation of *bhuR* expression in *B. avium* are ongoing.

**RhuR positively regulates  $P_{bhuR}$  expression.** To control expression of a specific gene, anti-sigma factors commonly bind to their cognate sigma factors, thereby denying productive interactions between the cognate sigma factors and RNAP (15). Genetic deletion of the anti-sigma factor gene releases the sigma factor from anti-sigma factor sequestration, enabling it to interact in an uncontrolled manner with RNAP and thus promoting constitutive expression of the regulated gene (9, 10, 19, 21, 32). To determine the means by which RhuR is involved in the heme induction cascade of *B. avium* and to determine if the polypeptide behaves as a typical sequestering type of anti-sigma factor, a chromosomal deletion of the *rhuR* ORF was engineered. Southern hybridization and colony PCR of 4169rif $\Delta rhuR$  confirmed that the deletion was confined to *rhuR* and that the nucleotide sequences encoding *rhuI*, *bhuR*, and the intergenic region located between *rhuR* and *bhuR*, which contains  $P_{bhuR}$ , were undisturbed (data not shown). The RhuR-dependent induction capacity of the wt and mutant strains of *B. avium* on  $P_{bhuR}$  was evaluated by introducing pDJM41, a reporter plasmid encoding a transcriptional fusion between  $P_{bhuR}$  and a promoterless *lacZ* gene, into 4169rif and 4169rif $\Delta rhuR$  (18). As shown previously by Kirby et al. (18), 4169rif(pDJM41, pRK415) exhibited minimal β-galactosidase activity when cultured in Fe-replete BHI broth and only slightly elevated β-galactosidase activity when cultured in Fe-limiting BHI broth (Fig. 2). Addition of 1 μM hemin to the Fe-limited BHI broth, however, elicited a dramatic increase in β-galactosidase reporter activity in 4169rif(pDJM41, pRK415). In contrast, when cultured under Fe-limited conditions regardless of the presence or absence of hemin supplementation, 4169rif $\Delta rhuR$ (pDJM41, pRK415) elaborated only low amounts of β-galactosidase activity (Fig. 2). When grown in Fe-replete or in Fe-stressed BHI broth, neither of the vector control strains [4169rif(pDJM41, pRK415) and 4169rif $\Delta rhuR$ (pDJM41, pRK415)] demonstrated significant differences in β-galactosidase reporter activities. These observations strongly indicated that 4169rif required RhuR for heme-dependent induction of  $P_{bhuR}$ .

To confirm that the induction deficit of  $P_{bhuR}$  was a consequence of the absence of *rhuR*, pAEK26, a recombinant plasmid that constitutively expresses wt *rhuR*, was conjugated into 4169rif $\Delta rhuR$ . While introduction of pAEK26 restored heme-dependent induction of  $P_{bhuR}$  in 4169rif $\Delta rhuR$ (pDJM41, pAEK26), the level of induction was not equivalent to the level of induction observed in the wt background [4169rif(pDJM41,

Ba RhuR	--MSAAGVLDLRRVAREARWMLHLASGQASADDAACERWRASDTRHEQAWQH----	AQR	54
Bp HurR	-----MAREAARWLVRLLGSGQASADEIQACDHWRASHAEHERAWQR----	ARR	44
Bb HurR	-----MAREAARWLVRLLGSGQASADEIQACDHWRASHAEHERAWQR----	ARR	44
Pp PupR	MNGQGATSI PGEVAEQAMHWHLELQEPVSAATLAACMSWRQAHPLHEHAWQRTQVFAQR		60
Ec FecR	-MNPLLTDSRRQALRSASHWYAVLSGERVSPQQEARWQQWYEQDQDNQAWWQQ----	VEN	55
	..* : * * . * . * . : : ***: . . .		
Ba RhuR	VNDLLGGLPQLLHATLGRPAKHTRRLALKAMAGMALGTPAAWAARWGRETGLLADYRS		114
Bp HurR	LTSMFDRIPPAVGQAALGR--ARRRAMLKSLVALLAAPPAAWAALRGARNSGWLADLRT		102
Bb HurR	LTSMFDRIPPAVGQAALGR--ARRRAMLKSLVALLAAPPAAWAALRGARNSGWLADLRT		102
Pp PupR	LREMRS PGQRPLAHAALRP--QQRRTALKQLSLLMAAGAGAWYLDKDAALVQDWRADYHS		118
Ec FecR	LRNQLGGVPGDVASRALHDT-RLTRRHVMKGLLLLLLGGAG-GGWQLWQSETGEGLRADYRT		113
	: . . : : * ** : * : : . . * . . ** :		
Ba RhuR	APGERRDIVLPGGTFVSLNLSGSDLDLSDGAVL---RAGEVYVR-----AGLRCVAHAP		165
Bp HurR	GTGETRTVALGPGTQLRLNTGTAVSLDDGSGLLSLRLHRGEIYLE-----ADRPCRVLTR		157
Bb HurR	GTGETRTVALGPGTQLRLNTGTAVSLDDGSGMLSLRLHRGEIYLE-----ADRPCRVLTR		157
Pp PupR	RIGEQRRLTLADGTQVQLNTDSALNVAFDQQARRLRLVRGEMLI TRPALADSRPLWVDTE		178
Ec FecR	AKGTVSRQQLLEDGSLTLTNTQSAADVRFDAHQRTVRLWYGEIAITAKDALQRPFVLRTR		173
	* * * : : ** : * . : . ** : :		
Ba RhuR	QGQVTASHARYALRLDAEGCRIEYVEGQVQVRPALGVAA--TLAAPQAACFDAYTVRLRS		223
Bp HurR	GGMIRTQAAHLWLRQDDADGLLGVVAGLAFWQGADNRTH--SVAAGQRIAWHDGALQGAA		215
Bb HurR	GGMIRTEAAHLWLRQDDADGLLGVVAGLAFWQGADNRTH--SVAAGQRIAWHDGALQGAA		215
Pp PupR	HGRLESTLAQFNVRHLHGQHTQATVYQGSVALQPALHAYPPILLGAGEQASFNQQLLARQ		238
Ec FecR	QGQLTALGTEFTVRQDNFTQLDQVQHAVEVLLASAPAKRIVNAGESLQFSASEFGAVK		233
	* : : : . : * . * . * : * : : . .		
Ba RhuR	AALESEPAWLRGVLRHVNAMPLAFAAEELGRYRRGLVRCQAQVAALPVSGTFQLDNTEGIL		283
Bp HurR	QTLDGSPDWLRGVLRADAMRLDRFLRELSRYRPGTLRCDPRVAGLRLSGVFQLAHTDDIL		275
Bb HurR	QTLDGSPDWLRGVLRADAMRLDHFLELSRYRPGTLRCDPRVAGLRLSGVFQLAHTDDIL		275
Pp PupR	AVAAVAPAWSQGMPLVAQQGPLAAFIEDLARYRRGHLACDPALAGLRVSGTFPLENTDKII		298
Ec FecR	PLDDESTSWTKDILSFSKPLGEVIATLTRYRNGVLRCDPAVAGLRLSGTFPLKNTDAIL		293
	. * : : * . * . * * * * : * : * . * : * * : * :		
Ba RhuR	RALPALLPVSVRSRTPYWIVIDKT-----		307
Bp HurR	RALPALLPVQLSYVTPYWITVGPRPAGATT		305
Bb HurR	RALPALLPVQLSYVTPYWITVGPRPAGATT		305
Pp PupR	AAVAETLQLEVQHFTRYVWTLKPRMA----		324
Ec FecR	NVIAQTLPVKIQSITRYWINISPL-----		317
	. . . * : : * * * :		

FIG. 1. Homology alignment of the RhuR protein of *B. avium* with HurR of *B. pertussis* (38), HurR of *B. bronchiseptica* (38), FecR of *E. coli* (39), and PupR of *P. putida* (19). Amino acids are denoted using the one-letter code. Ba, *B. avium*; Bp, *B. pertussis*; Bb, *B. bronchiseptica*; Ec, *E. coli*; Pp, *P. putida*. \*, amino acids which are conserved in all five proteins; colon, amino acids with similar side chains; -, gaps in the amino acid sequences in comparison to the other four proteins. Tryptophans which are conserved in all five proteins and which have been shown to be essential for induction in *E. coli* FecA (35) are denoted in boldface and larger font. Amino acid sequences of HurR from *B. pertussis* and *B. bronchiseptica* were taken from the National Center for Biotechnology Information website (accession numbers NP879221 and NP891186, respectively).

pRK415)] (Fig. 2). Observations in other systems suggested a possible explanation for this conundrum. Proper functioning of the CarQ/CarR sigma factor system in *M. xanthus* is exquisitely sensitive to differences in stoichiometric expression of CarQ and CarR (10). Several factors suggest that the expression of RhuI and RhuR are similarly coupled: *rhuI* and *rhuR* are encoded on a polycistronic message and are likely to be transcriptionally linked (A. E. Kirby and T. D. Connell, data not shown); *rhuI* and *rhuR* have a single codon overlap, a motif that is commonly observed in genes which are coupled at the level of translation (10). Thus, it is not unreasonable to hypothesize that, similar to CarQ and CarR, optimal functioning of RhuR and RhuI required close stoichiometric expression of both proteins. If that model is correct, the failure of pAEK26 to fully complement the *rhuR* mutant could be explained as a

consequence of stoichiometric imbalance brought about by overexpression of the plasmid-encoded *rhuR* relative to the endogenous expression of the chromosomally encoded *rhuI*. To test this model, pAEK26 was introduced into 4169rif (pDJM41), a strain in which the chromosomal copies of *rhuI* and *rhuR* were undisturbed. Introduction of pAEK26 did not significantly alter the inductive capacity of  $P_{bhuR}$  in the wt strain (data not shown). Thus, it is doubtful that the inability of pAEK26 to reestablish equivalent expression levels in the *rhuR* mutant was a result of a disparity in gene dosage between *rhuI* and *rhuR*.

Nevertheless, it was evident that efficient heme-dependent induction of  $P_{bhuR}$  in *B. avium* required RhuR. Furthermore, as the deletion of *rhuR* did not elicit constitutive expression from the RhuI-dependent  $P_{bhuR}$  in 4169rif $\Delta$ *rhuR*, these exper-

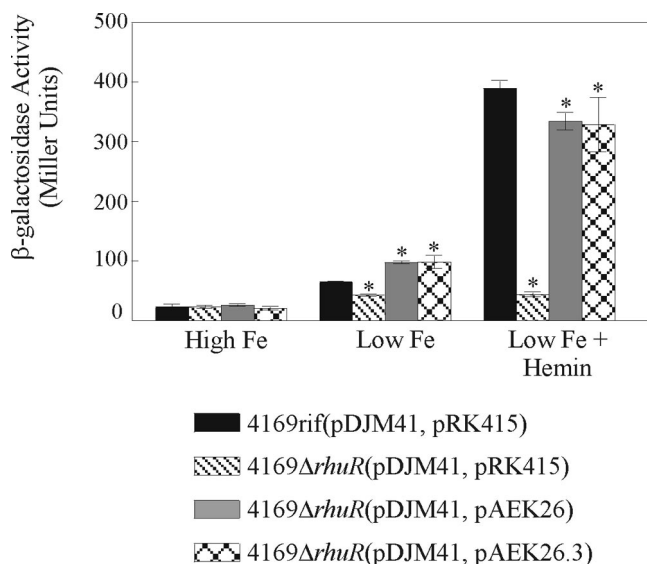


FIG. 2. RhuR is a positive regulator of the BhuR-dependent heme induction cascade of *B. avium*. 4169rif(pDJM41, pRK415), 4169rifΔrhuR(pDJM41, pRK415), 4169rifΔrhuR(pDJM41, pAEK26), and 4169rifΔrhuR(pDJM41, pAEK26.3) were cultured in BHI broth supplemented with 100 μM EDDHA to deplete the internal Fe stores. Fe-stressed cultures were used to inoculate high-Fe (BHI plus 150 μM FeSO<sub>4</sub>), low-Fe (BHI plus 100 μM EDDHA) and low-Fe plus hemin (BHI plus 100 μM EDDHA plus 1 μM hemin) broth cultures. After 16 h of incubation, the β-galactosidase activity expressed from pDJM41 was determined using a modified Miller assay (27). Results shown represent the average of three experiments. Error bars indicate ±1 standard deviation. An asterisk indicates a significant difference ( $P < 0.05$ ) from 4169rif(pDJM41, pRK415) under the same condition, as determined by Student's *t* test.

iments strongly implied that RhuR was not a sequestering type of anti-sigma factor. Rather, RhuR appeared to be a positive activator of  $P_{bhuR}$  expression.

**RhuR is a cytoplasmic membrane protein.** To functionally bridge the outer membrane protein BhuR and the cytoplasmic protein RhuI, RhuR was predicted to be localized either to the cytoplasmic membrane or to the periplasm. Evidence of a putative signal peptide in RhuR supported this model. Additional evidence to support a cytoplasmic membrane localization of RhuR was provided by computer-based polypeptide analysis (TMHMM [<http://www.cbs.dtu.dk/services/TMHMM-2.0/>], TMPred [[http://www.ch.embnet.org/software/TMPRED\\_form.html](http://www.ch.embnet.org/software/TMPRED_form.html)], TopPred2 [<http://www.sbc.su.se/~erikw/toppred2/>], and DAS [<http://www.sbc.su.se/~miklos/DAS/>]). The presence of a single membrane-spanning region (amino acids 81 to 100) with hydrophobic character was predicted by TMHMM. Three additional membrane-spanning regions (amino acids 199 to 219, 232 to 252, and 257 to 276) were predicted by TMPred, and two putative membrane-spanning regions were predicted by TopPred2 (amino acids 121 to 141 and 198 to 218). Somewhat paradoxically, analysis by DAS indicated that RhuR lacked motifs consistent with strong transmembrane domains. To resolve these contradictory predictions, fractionation experiments were conducted to directly establish the location of RhuR in the cell. Since antiserum to the wt RhuR was not available for use in these experiments, an alternative strategy was employed in which the cellular location of a His-tagged version of RhuR was ascertained. To verify that fusion of the

His<sub>6</sub> tag to the carboxyl terminus of RhuR did not interfere with proper localization of the protein, pAEK26.3, a plasmid encoding a His<sub>6</sub>-tagged version of RhuR, was introduced into 4169rifΔrhuR(pDJM41). Genetic complementation experiments confirmed that introduction of pAEK26.3 into 4169rifΔrhuR(pDJM41) restored heme-dependent expression from  $P_{bhuR}$  to a level which was essentially equivalent to that observed in the mutant strain complemented with pAEK26 encoding wt rhuR [Fig. 2, 4169rifΔrhuR(pDJM41, pAEK26) versus 4169rifΔrhuR(pDJM41, pAEK26.3)]. Since it is likely that proper localization of the protein in the cell is essential for regulatory activity, this experiment indicated that the His<sub>6</sub>-tagged protein was transported to its active cellular location.

To localize RhuR-His<sub>6</sub> in the cell, proteins in total membranes, outer membranes, and cytoplasmic membranes isolated from hemin-induced cultures of 4169rifΔrhuR(pDJM41, pRK415) and 4169rifΔrhuR(pDJM41, pAEK26.3) were resolved by SDS-PAGE and immunoblotted for poly-His epitopes. Western blotting with INDIA HisProbe-HRP (Pierce) revealed a strongly reactive polypeptide with an apparent molecular mass of 30 kDa in total membranes obtained from 4169rifΔrhuR(pDJM41, pAEK26.3) which was absent in total membranes isolated from the vector control (Fig. 3). Total membranes were further fractionated by extraction with 1% lauroyl sarcosine, which dissolves the cytoplasmic membrane but not the outer membrane. INDIA HisProbe-reactive protein was observed only in the cytoplasmic membrane fraction and not in the outer membrane fraction from 4169rifΔrhuR(pDJM41, pAEK26.3) (Fig. 3). Polymyxin B sulfate extracts of whole cells analyzed using similar blotting strategies demonstrated that the INDIA HisProbe-reactive protein of 4169rifΔrhuR(pDJM41, pAEK26.3) was not a component of the periplasm (data not shown). These cell fractionation experiments provided strong evidence that RhuR is either an integral

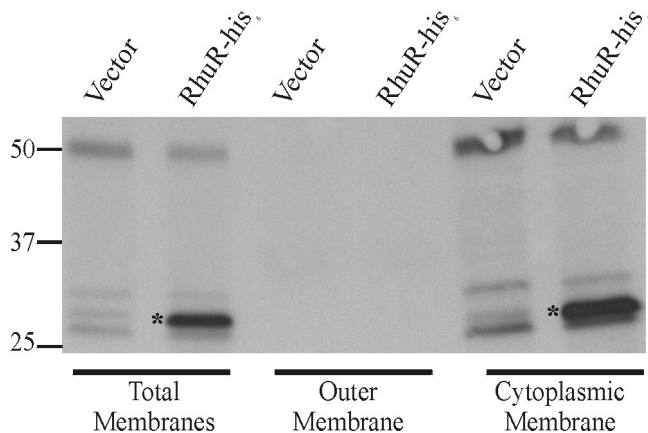


FIG. 3. RhuR-His<sub>6</sub> is localized to the inner membrane. Fe-stressed cultures of 4169rifΔrhuR(pDJM41, pRK415) (Vector) and 4169rifΔrhuR(pDJM41, pAEK26.3) (RhuR-his<sub>6</sub>) were used to inoculate 500 ml BHI plus 100 μM EDDHA plus 1 μM hemin. At stationary phase, cells were isolated for preparation of total membranes, outer membranes, and cytoplasmic membranes. Twenty micrograms of total protein from each membrane preparation was resolved by SDS-PAGE using SDS-12% polyacrylamide gels. Proteins were transferred to nitrocellulose, and His-tagged RhuR was detected by Western blotting by use of the INDIA HisProbe-HRP. The positions of the molecular mass markers (in kilodaltons) are indicated. Asterisks indicate the position of RhuR-his<sub>6</sub>.

cytoplasmic membrane protein or is tightly associated with the cytoplasmic membrane. These observations were consistent with the structural predictions obtained from the computer analysis of the predicted RhuR polypeptide.

**The N terminus of RhuR contains the activation domain.** To understand the precise molecular interactions which mediate RhuR-dependent regulation of RhuI, it will be essential that the functionally important domains in these proteins are mapped. To begin to map the region(s) of RhuR required for heme-dependent activation of  $P_{bhuR}$ , a series of fusion proteins was engineered. pAEK21 encodes a chimera in which the N-terminal 97 amino acids of RhuR, inclusive of the native signal peptide, is genetically fused to BlaM. As expected, growth of 4169rif $\Delta$ rhuR(pDJM41, pAEK21) in Fe-replete (150  $\mu$ M FeSO<sub>4</sub>) culture broth induced only minimal  $\beta$ -galactosidase activity (Fig. 4B). Surprisingly, 4169rif $\Delta$ rhuR(pDJM41, pAEK21) cultured under conditions of Fe stress, but in the absence of hemin, exhibited levels of  $P_{bhuR}$  reporter activity similar to that observed for hemin-induced, Fe-stressed wt cells [4169rif(pDJM41, pRK415)] (Fig. 4A versus B). Addition of hemin to the Fe-stressed cultures of 4169rif $\Delta$ rhuR(pDJM41, pAEK21) did not further increase  $P_{bhuR}$  activity (Fig. 4B). These results are consistent with a model in which the RhuR-BlaM chimera constitutively activates the  $P_{bhuR}$  promoter in Fe-stressed cells. In effect, the RhuR-BlaM fusion decoupled  $P_{bhuR}$  from hemin-dependent induction. The observation that the effect could be elicited only under Fe-stressed conditions suggested that chimera-dependent decoupled induction required expression of Fe-repressible RhuI. To determine if the chimera exerted its activity in a wt background, pAEK21 was also introduced into 4169rif(pDJM41). Under both Fe-stressed and hemin-induced conditions, 4169rif(pDJM41, pAEK21) exhibited higher  $P_{bhuR}$  activity than did the relevant vector control [Fig. 4A, 4169rif(pDJM41, pAEK21) versus 4169rif(pDJM41, pRK415)] under similar growth conditions. The effect was also hemin inducible. Thus, wt RhuR is dominant over the chimera in respect to RhuI-dependent, hemin-dependent induction of  $P_{bhuR}$ .

As a control to demonstrate that the decoupling activity of the RhuR-BlaM fusion was a property of the N-terminal 97 amino acids of RhuR and not to the BlaM portion of the chimera, a second fusion was engineered. In this new recombinant plasmid (pAEK23), a genetic fusion was created between the N-terminal 97 amino acids of the polypeptide and 18 irrelevant amino acids encoded by plasmid vector sequences. As shown in Fig. 4,  $P_{bhuR}$  activity of this 115-amino-acid chimera was similar to that of the RhuR-BlaM fusion, indicating that the decoupled regulatory activity was engendered solely by the N-terminal region of RhuR and was not influenced by the presence of BlaM.

Prior cellular fractionation experiments indicated that wt RhuR localized to the cytoplasmic membrane. To investigate the cellular localization of the RhuR-BlaM chimera, cells expressing the chimera were fractionated into total membranes, outer membranes, and cytoplasmic membranes, each of which was analyzed by SDS-PAGE and immunoblotting using anti-BlaM antiserum. Anti-BlaM Western blotting of total membranes from the vector control, 4169rif $\Delta$ rhuR(pDJM41, pRK415), revealed three cross-reactive polypeptides (Fig. 5). When equal amounts of protein from total membranes of 4169rif $\Delta$ rhuR(pDJM41, pAEK21) were probed with anti-

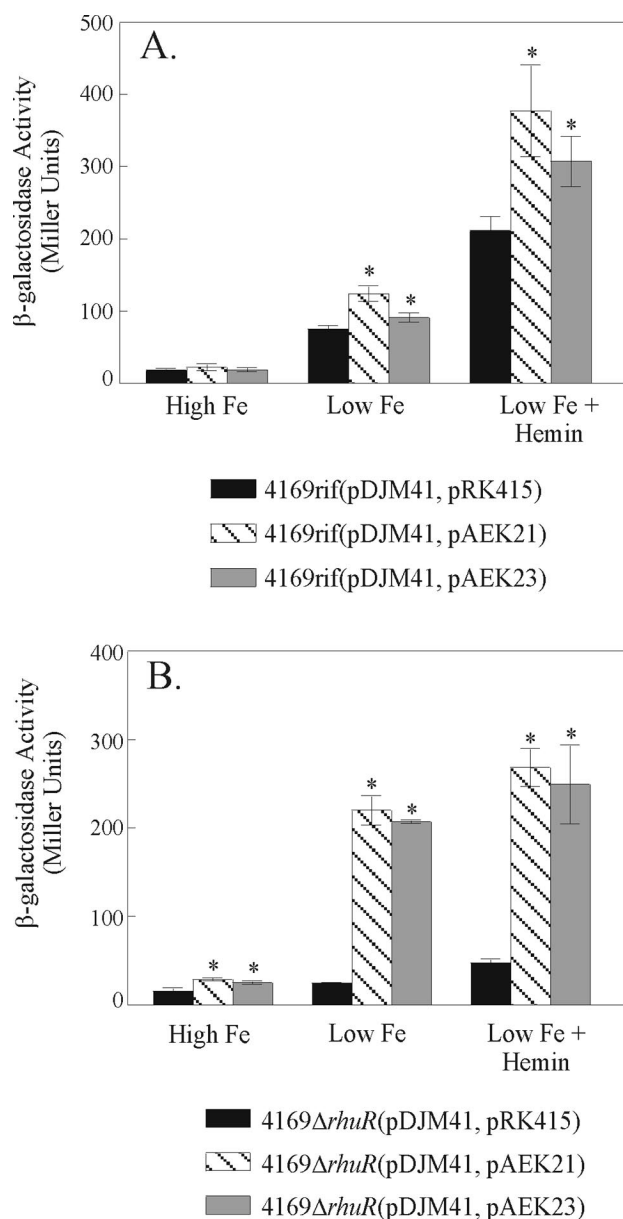


FIG. 4. The N terminus of RhuR activates  $P_{bhuR}$  in the absence of hemin. Fe-stressed overnight cultures were used to inoculate high-Fe (BHI plus 150  $\mu$ M FeSO<sub>4</sub>), low-Fe (BHI plus 100  $\mu$ M EDDHA), and low-Fe plus hemin (BHI plus 100  $\mu$ M EDDHA plus 1  $\mu$ M hemin) broth cultures. After 16 h of incubation, the  $\beta$ -galactosidase activity of the cultures was determined using a modified Miller assay (27). (A) Activity of the RhuR fusions in wt *B. avium*. pAEK21 expresses a chimeric protein consisting of the first 97 amino acids of RhuR fused to  $\beta$ -lactamase (BlaM); pAEK23 encodes the same 97-amino-acid region of RhuR fused to 18 irrelevant amino acids encoded by the vector. (B)  $P_{bhuR}$  promoter activity of the RhuR fusions in 4169rif $\Delta$ rhuR. Numbers represent the average  $\beta$ -galactosidase activity of triplicate cultures. Error bars indicate  $\pm 1$  standard deviation. Asterisks indicate a significant difference ( $P < 0.05$ ) from the vector control cultured under the same condition, as determined by Student's *t* test.

BlaM, two additional immunoreactive proteins that migrated to 37 and 25 kDa were apparent (Fig. 5). The 37-kDa species corresponded with the predicted molecular mass of RhuR-BlaM. As it was only seen in membrane fractions from

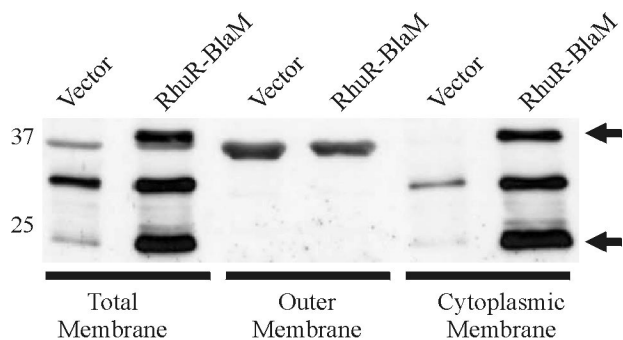


FIG. 5. RhuR-BlaM is localized to the inner membrane of 4169rif $\Delta$ rhuR. Fe-stressed cultures of 4169rif $\Delta$ rhuR(pDJM41, pRK415) (vector) and 4169rif $\Delta$ rhuR(pDJM41, pAEK21) (RhuR-BlaM) were used to inoculate BHI broth supplemented with 100  $\mu$ M EDDHA and 1  $\mu$ M hemin. At stationary phase, cells were collected for isolation of total membranes, outer membranes, and cytoplasmic membranes. Ten micrograms of total protein from each membrane preparation was separated by SDS-PAGE using SDS-12% polyacrylamide gels. Western immunoblotting using a rabbit polyclonal anti-BlaM antiserum was used to detect immunoreactive proteins. The positions of the molecular mass markers (in kilodaltons) are indicated. Arrows indicate the position of the 37-kDa RhuR-BlaM and its major 25-kDa degradation product.

4169rif $\Delta$ rhuR(pDJM41, pAEK21), the 25-kDa species was hypothesized to be a degradation product of RhuR-BlaM. Fractionations of the total membranes demonstrated that a set of immunoreactive proteins, which were identical in electrophoretic mobility to those in total membranes, were expressed in the cytoplasmic membrane fraction (Fig. 5). Only a vector-specific band was detected in the outer membrane (and in the periplasmic extract) (data not shown) of 4169rif $\Delta$ rhuR(pDJM41, pAEK21). These experiments provided clear evidence that the RhuR-BlaM chimera, and also wt RhuR, localized to the cytoplasmic membrane of *B. avium*.

The inductive capacity of the RhuR-BlaM chimera suggested a model for RhuR activity. Since expression of RhuR-BlaM activates  $P_{bhuR}$ , it can be surmised that the N-terminal 97 amino acids of RhuR likely encode the residues which are required for activation of RhuI. Furthermore, since activation of the cytoplasmic RhuI likely requires interaction between RhuI and RhuR, at least a portion of the N-terminal 97 amino acids of RhuR are likely exposed to the cytoplasm. Extrapolative interpretation of that model implies that the C-terminal portion of RhuR (amino acids 98 to 307) is exposed to the periplasm and encodes the *cis*-acting heme-dependent (i.e., BhuR-dependent) domain(s) that regulates the N-terminal activation portion of the protein. Chimeras for expression in *B. avium* in which the carboxyl portion of RhuR is to be genetically fused to MalE are being engineered to experimentally test this model.

**Expression of BhuR and growth of *B. avium* in hemin does not require RhuR.** Expression of BhuR is essential for optimal growth of *B. avium* in culture medium in which hemin or hemoproteins are the sole sources of nutrient Fe (28). Having shown that RhuR was required to upregulate expression of  $P_{bhuR}$ , it was hypothesized that RhuR was also required for efficient growth of *B. avium* in culture medium in which hemin was the Fe-limiting nutrient source. Previous endpoint growth assays demonstrated that growth of *B. avium* is inhibited when cultured in BHI broth depleted of available Fe by the addition of 400  $\mu$ M EDDHA (18, 28). Supplementation of BHI plus 400  $\mu$ M EDDHA broth with 300  $\mu$ M FeSO<sub>4</sub> rescued growth of the cells (28) (Fig. 6, FeSO<sub>4</sub>). To our surprise, when 4169rif $\Delta$ rhuR was cultured in Fe-deficient BHI broth supplemented with either 5  $\mu$ M hemin (data not shown) or 1.25  $\mu$ M hemoglobin (Fig. 6), growth of the mutant was indistinguishable from growth of the isogenic parental strain (28). To examine this apparent conundrum, the outer membrane protein profile of 4169rif $\Delta$ rhuR was examined to compare the expression of BhuR in the mutant strain with expression of BhuR in the wt

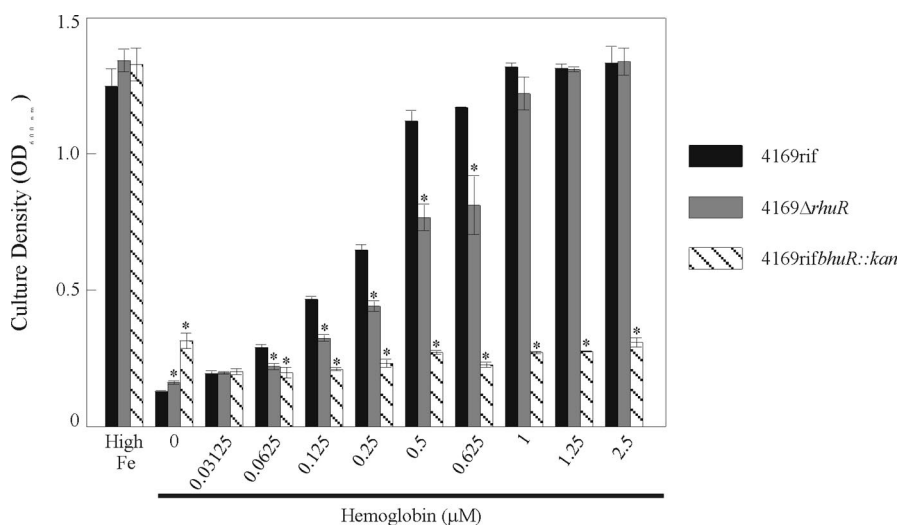


FIG. 6. RhuR is required by *B. avium* for efficient utilization of hemoglobin as source of nutrient Fe. Fe-stressed cultures of 4169rif, 4169rifbhuR::kan, and 4169rif $\Delta$ rhuR were used to inoculate 2 ml of BHI broth which was supplemented with 400  $\mu$ M EDDHA and 300  $\mu$ M FeSO<sub>4</sub> (High Fe) or with turkey hemoglobin at the indicated concentrations. After 24 h of incubation at 37°C, the OD<sub>600</sub> of the cultures was measured. Results represent the average cell densities of three cultures. The heme acquisition mutant 4169rifbhuR::kan was used as a negative control (28). Error bars indicate  $\pm$ 1 standard deviation. Asterisks indicate a statistically significant difference from 4169rif (Student's *t* test).



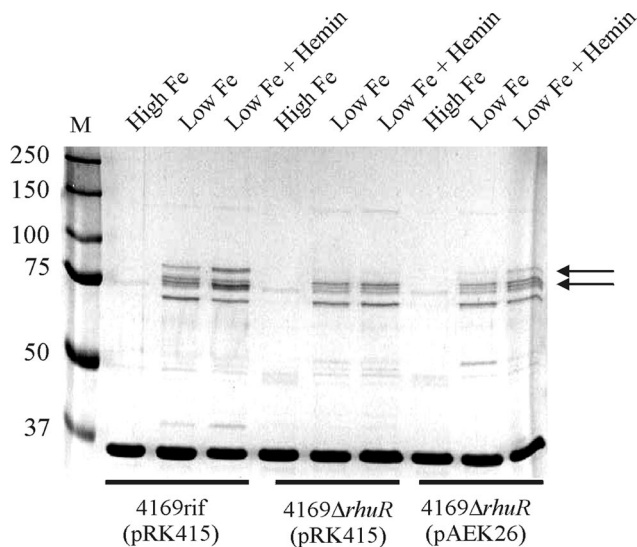


FIG. 7. BhuR is expressed at low levels in the outer membrane of 4169rif $\Delta$ *rhuR*. Fe-stressed (BHI plus 100  $\mu$ M EDDHA), Fe-stressed plus heme (BHI plus 100  $\mu$ M EDDHA plus 1  $\mu$ M hemin), and Fe-replete (BHI plus 150  $\mu$ M FeSO<sub>4</sub>) cultures of 4169rif(pRK415), 4169rif $\Delta$ *rhuR*(pRK415), and 4169rif $\Delta$ *rhuR*(pAEK26) were used for isolation of outer membranes. The proteins in the outer membrane preparations were resolved in an SDS–8.75% polyacrylamide gel. Protein profiles were visualized by staining with colloidal Coomassie brilliant blue. M, molecular mass markers (in kilodaltons). Arrows indicate the positions of the 91-kDa and 82-kDa forms of BhuR (28).

parent strain. Contrary to expectations, expression of the two forms of BhuR (91 and 82 kDa) (18, 28) was not inhibited in the *rhuR* mutant strain [Fig. 7, 4169rif $\Delta$ *rhuR*(pRK415), low Fe and low Fe plus hemin]. Expression of BhuR in 4169rif $\Delta$ *rhuR* (pRK415), however, was somewhat lower than expression of the protein in 4169rif(pRK415) when both strains were cultured under conditions of Fe stress in the absence of hemin induction [Fig. 7, 4169rif $\Delta$ *rhuR*(pRK415) versus 4169rif(pRK415), both in low Fe]. When hemin was added to the Fe-stressed cultures, expression of the two forms of BhuR was enhanced in the wt strain [Fig. 7, 4169rif(pRK415), low Fe, versus 4169rif(pRK415), low Fe plus hemin]. Expression of BhuR was unaffected in the mutant strain [Fig. 7, 4169rif $\Delta$ *rhuR* (pRK415), low Fe versus 4169rif $\Delta$ *rhuR*(pRK415), low Fe plus hemin] when cultured in low Fe plus hemin. Further analysis showed that, while 4169rif $\Delta$ *rhuR*(pRK415), in comparison to the isogenic wt strain [4169rif(pRK415)], was incapable of hemin-dependent upregulation of BhuR expression, complementation of 4169rif $\Delta$ *rhuR* with pAEK26 (i.e., with wt *rhuR*) restored the ability of the mutant to upregulate expression of BhuR in a hemin-dependent manner [Fig. 7, 4169rif $\Delta$ *rhuR* (pAEK26), low Fe and low Fe plus hemin]. It should be noted that the low level expression of BhuR in wt *B. avium* in the absence of hemin is promoted, at least in part, by read-through transcription from the upstream Fur-dependent P<sub>*rhuR*</sub> (unpublished results).

During a natural infection, the various sequestration and compartmentalization mechanisms operating in the host reduce the physiological concentrations of heme or hemoglobin to levels considerably below the 5  $\mu$ M used in the prior *in vitro* growth experiments. With this point in mind, it was surmised

that the failure of the *rhuR* mutant to demonstrate a detectable growth defect in comparison to wt *B. avium* was a result of the use of physiologically irrelevant amounts of hemin in the broth cultures. Experiments, therefore, were repeated using lower concentrations of hemin and hemoglobin to address this issue. As a control to establish a baseline for hemin- and hemoglobin-limited growth of the bacterium, 4169rif*bhuR::kan*, a mutant deficient in expression of *bhuR*, was included in these growth experiments. As expected, the growth of 4169rif*bhuR::kan* was significantly inhibited under all conditions with the exception of culture broth supplemented with FeSO<sub>4</sub> (Fig. 6). Growth of 4169rif $\Delta$ *rhuR*, however, was indistinguishable from that of 4169rif when each strain was cultured in BHI broth without hemin supplementation or in BHI broth supplemented with hemin. This effect was observable at all concentrations of hemin between 0.125 and 10  $\mu$ M in a twofold dilution series (data not shown). In contrast, a significant difference in Fe substrate utilization was observed when hemoglobin was substituted for hemin in the broth cultures. While dose-dependent growth of 4169rif was evident in BHI broth containing hemoglobin at concentrations as low as 0.0625  $\mu$ M, dose-dependent growth of 4169rif $\Delta$ *rhuR* was not evident until the concentration of hemoglobin in the broth reached 0.125  $\mu$ M (Fig. 6). Furthermore, the difference in dose-dependent hemoglobin utilization between the *rhuR* mutant and the wt strain remained significant from 0.0625 to 0.625  $\mu$ M hemoglobin in the broth. At hemoglobin concentrations exceeding 1.0  $\mu$ M, no significant difference was observed between the growth of the *rhuR* mutant and the wt strain.

## DISCUSSION

During invasion of vertebrate hosts, pathogenic bacteria are normally confronted with a stringently Fe-limited environment. Without mechanisms to efficiently procure Fe from the cells, fluids, and tissues of the infected host, most bacteria are incapable of expressing full virulence (13, 28). Since Fe is toxic to the bacterial cell when intracellular concentrations are uncommonly high, expression of Fe uptake mechanisms must be rigidly controlled. It is critical, therefore, that bacteria balance nutritional needs against potential toxicity by tightly regulating the active uptake of Fe. While various Fe-dependent bacterial regulators are currently known, only Fur, a global Fe-dependent repressor of Fe uptake systems, has been extensively studied. In most cases, Fur represses transcription of Fur-dependent genes when the cellular concentration of Fe is optimal (7). When concentrations of Fe within the cell fall to critical levels, Fur-dependent Fe uptake genes are derepressed. This system of negative regulation ensures that Fur-regulated Fe acquisition systems are expressed only when the cell has a critical need for the metal.

Most bacteria exhibit multiple Fur-regulated Fe acquisition systems, each of which targets one or more forms of inorganic or organic Fe as a nutrient source. Expression by the bacterium of these systems solely in response to Fe stress in the absence of those specific Fe-containing substrates in the local environment potentially wastes precious energy and metabolic resources. In response to that selective pressure, bacteria evolved complicated regulatory systems to fine-tune the expression of some Fur-dependent Fe uptake mechanisms. The evolution or

acquisition of the Fur- and heme-dependent Bhu uptake system in *B. avium* likely arose as an adaptive response to the abundance of heme and hemoproteins in the avian host (28). It is becoming increasingly evident that the regulation of the Bhu system of *B. avium* is a complicated regulatory cascade which responds both to Fe stress and the presence of heme and hemoglobin (18, 28). In Fe-replete cells, transcription of the *bhuRSTUV* operon is repressed by Fur (18). Upon Fe starvation, the upstream Fur-dependent  $P_{rhuR}$  is derepressed with the subsequent transcription of *rhuI* and *rhuR*. Concomitant with transcription of *rhuI* and *rhuR* is a low level expression of BhuR in the outer membrane of the Fe-stressed bacterium. This low level of expression of BhuR is a consequence of incomplete termination of *rhuIR* transcription, which gives rise to a rare polycistronic mRNA that encodes *rhuI*, *rhuR*, and *bhuR* (unpublished data). This system of read-through transcription ensures that Fe starvation of *B. avium* not only elicits optimal expression of RhuI and RhuR but also produces a low level of BhuR expression sufficient to prime the cell for upregulation of the entire *bhuRSTUV*-encoded heme uptake system, should heme become locally available. Upregulation of the regulatory cascade initiates by binding of trace molecules of heme by BhuR. Binding to BhuR transduces a signal across the periplasm to RhuR. The signal is propagated through RhuR across the cytoplasmic membrane to RhuI, which is localized in the cytoplasm. Upon reception of the signal, RhuI directly or indirectly escorts RNAP to  $P_{bhuR}$ , thus promoting high-level transcription of *bhuR* and the other four genes of the polycistron (*bhuSTUV*). This heme-dependent, three-component regulatory system parsimoniously ensures that *B. avium* expresses the heme utilization mechanism only when two conditions are met: (i) when the cell is starved for Fe and (ii) when either heme or hemoproteins are available in the local environment.

It is clear that the mechanism for heme uptake in *B. avium* is governed by a precise molecular interplay between the ECF sigma factor (RhuI) and the cytoplasmic membrane activator (RhuR). Many traditional anti-sigma factors control the function of their cognate sigma factor by sequestering the sigma factor from core RNAP (15). In those cases, genetic deletion of the anti-sigma factor typically releases the sigma factor from regulatory control, which manifests as constitutive expression of the sigma factor-dependent regulated gene (9, 10, 19, 21, 32). Other sigma factor regulators exert control of their cognate sigma factors via various molecular interactions, including phosphorylation-dependent partner switching (5), extracellular export of the anti-sigma factor (14), and a regulated degradation of the anti-sigma factor (1, 10). In this study, deletion of *rhuR* did not elicit a constitutive deregulation of the RhuI-dependent  $P_{bhuR}$ . Rather, deletion of *rhuR* inhibited heme-dependent induction of  $P_{bhuR}$ , thereby providing strong evidence that RhuR is not a traditional sequestering type of anti-sigma factor but is a positive regulator of RhuI activity. Similar sigma factor activation mechanisms have been reported in other bacteria. Genetic experiments revealed that FecR, an *E. coli* homologue of RhuR, is required for transcriptional activity of FecI, its cognate ECF sigma factor (30). Restoration of  $P_{fecA}$  activity was not established when a *fecIR-fecABCDE* null strain was complemented solely with a plasmid encoding *fecA* (the outer membrane ferric citrate receptor) and *fecI* (the ECF sigma factor). Promoter activity was re-

stored only when the *fecIR-fecABCDE* null strain was complemented with *fecA*, *fecI*, and *fecR* (30). While studies confirmed that FecR directly interacts with FecI (6, 25), the molecular mechanism by which FecI is activated, or the nature of the difference between the functional structures of activated and inactivated FecI, have not been well elucidated. The successful mapping of the activator domain of RhuR reported herein will likely facilitate the elucidation of the molecular mechanisms by which RhuI is biochemically or biophysically activated.

Using cells expressing chromosomally encoded and plasmid-encoded truncated FecR derivatives (30) and plasmid-encoded FecR'-BlaM hybrid proteins (43), it was demonstrated that truncated forms of FecR as small as 59 residues had the capacity to stimulate ferric citrate-independent expression of the *fecA* and *fecB* transport genes. It was later confirmed using a bacterial two-hybrid Lex-based system (6) that the N-terminal portion of RhuR directly interacts with RhuI. Expression of the N-terminal 97 amino acids of RhuR decouples the heme dependency of  $P_{bhuR}$  from the heme induction cascade in *B. avium*. Fusions of these 97 amino acids to either BlaM or to 18 vector-encoded irrelevant amino acids constitutively stimulated  $P_{bhuR}$  activity in cells cultured under Fe-stressed conditions in the absence of hemin to levels which were equivalent to those observed in Fe-starved cells cultured in the presence of hemin. These data support a model in which the N-terminal 97-amino-acid region of RhuR interacts productively with cytoplasmic RhuI. This region, therefore, has been designated as the activator domain of the protein. The fact that activation via RhuR requires heme-dependent transduction from BhuR suggests that RhuR must also harbor a controller domain for intercepting this transduction signal. Experiments in *E. coli* revealed that the C-terminal region of FecR is located in the periplasm (43) and interacts productively with FecA (6). Using the Fec system as a model, we hypothesize that the C-terminal region of RhuR is periplasmically exposed and that this region is the controller domain, which receives the heme-dependent transducing signals from BhuR. In general, we anticipate that interaction of heme-bound BhuR with the C-terminal region of RhuR elicits a conformational change in RhuR, which is translated across the cytoplasmic membrane to the N terminus of RhuR. A concomitant conformational change in the N-terminal region of RhuR elicits, directly or indirectly, a heme-dependent activation of RhuI. Future studies will investigate these putative interactions between BhuR and the C-terminal region of RhuR and between the N-terminal region of RhuR and RhuI and the structural changes in each protein that occur.

It should be noted that our domain model of RhuR is based upon the hypothesis that the decoupling activity of the RhuR chimera is due to the separation of its activator domain from its controller domain. There is, however, an alternative model that could explain the manner in which the chimeras decouple  $P_{bhuR}$  from heme-dependent regulation. Decoupling could simply be a result of the overexpression of the chimera, which disturbs a regulatory equilibrium in the cell. We discount this alternative model, since overexpression of a plasmid-encoded wt *rhuR* in Fe-stressed wt cells does not elicit a detectable change in  $P_{bhuR}$  activity (data not shown).

The establishment of the essential nature of BhuR for heme uptake and the corresponding dependence of RhuR for opti-

mal BhuR expression provide solid evidence to support the hypothesis that RhuR is required for efficient growth in culture medium in which heme is the sole source of nutrient Fe. To our surprise, endpoint growth assays demonstrated that, regardless of heme concentration, growth of *B. avium* in culture broth in which heme was the limiting Fe source did not require expression of RhuR. The capacity to use hemoglobin as a sole source of Fe, however, was strongly correlated with expression of RhuR. This response was particularly evident at lower hemoglobin concentrations, which may be indicative of the actual substrate likely encountered by *B. avium* during a natural infection. Free heme is rarely found in the fluids and tissues of infected hosts. Hemoglobin, however, becomes quite abundant as the bacterium degrades tissues in the upper respiratory system. It is not surprising, therefore, that *B. avium* evolved a system to rapidly access the concentration of hemoglobin, the most likely form of organic Fe, and used the sensing system to finely coordinate the regulated expression of the proteins involved in its acquisition. Based on the ability of *B. avium* to efficiently utilize hemoglobin as an Fe source, we predicted the existence of a molecule which can extract or release the heme moiety from hemoglobin and from other hemoproteins (28). A logical assumption would be that the bacterium produces a protease that degrades hemoglobin, thus releasing heme into the medium. In *E. coli*, for example, hemoglobin is degraded by a secreted protease (31). Using sensitive hemoglobin-specific immunoblotting, we have been unable to demonstrate a similar proteolytic activity in *B. avium*. The molecular mechanism by which heme is obtained from hemoglobin by *B. avium* warrants further investigation.

Comparisons between the *rhuIR* and *bhuR* genes and their respective protein products have been informative in substantiating the importance of the genes in Fe acquisition in the bordetellae. The *rhuIR-bhuR* locus is conserved at the nucleotide and amino acid levels and in genetic organization throughout the genus *Bordetella*. RhuI of *B. avium* is 65% similar to the HurI of *B. pertussis* and 83% similar to the HurI of *B. bronchiseptica* (38). Similarly, the BhuR of *B. pertussis* is 64% similar to the BhuR of *B. avium*. BhuRs from *B. bronchiseptica* and from *B. parapertussis* are 81% similar with the BhuR of *B. avium*. A very strong selective pressure is required to maintain these significant levels of similarity between RhuI and BhuR among the four virulent species of *Bordetella*. Complementation experiments are being performed to determine whether BhuR and RhuI of *B. avium* are true functional homologues in these species.

In contrast to the high degree of conservation of BhuR and RhuI, RhuR is more divergent among the *Bordetella* species. HurR (the RhuR homologue) of *B. bronchiseptica* and HurR of *B. pertussis* are both only 51% similar to RhuR of *B. avium* (Fig. 1). Perhaps the most interesting member of the RhuR family is encoded by *B. parapertussis*. While the first 83 amino acids of RhuR of *B. parapertussis* are 100% identical to the corresponding region of HurR of *B. pertussis*, the remaining 151 amino acids of the *B. parapertussis* protein have little or no homology with the RhuR polypeptides of *B. avium* or to either of the other two *Bordetella* species. Perhaps the heme-dependent induction system in *B. parapertussis* has been rendered nonfunctional. There is, however, another intriguing possibility. It is the N-terminal region of the *B. parapertussis* RhuR that

is highly conserved. That region in RhuR of *B. avium* was shown in this study to harbor the RhuI activation domain. And, when that domain was separated from the putative C-terminal controller domain, the polypeptide stimulated expression of  $P_{bhuR}$  in a heme-independent manner. Thus, it is possible that RhuR of *B. parapertussis* constitutively activates its cognate RhuI when exposed to Fe starvation, regardless of heme availability. Although eliminating heme-dependent control of heme uptake, such a decoupled system would, nevertheless, enable *B. parapertussis* to maximally express BhuR and presumably the other genes of the *bhu* locus. Whether such a decoupled system in *B. parapertussis* would be energy efficient or selectively adaptive is unclear.

Experiments detailed in this report provide strong evidence that expression of RhuR is essential for heme-dependent induction of BhuR and that the regulatory protein is required by *B. avium* for efficient utilization of hemoglobin as a source of nutrient Fe. Future studies will examine the function of RhuR throughout the *Bordetella* genus.

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