

Use of Heme-Protein Complexes by the *Yersinia enterocolitica* HemR Receptor: Histidine Residues Are Essential for Receptor Function

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The abilities of two bacterial active heme transporters, HmbR of *Neisseria meningitidis* and HemR of *Yersinia enterocolitica*, to use different heme sources were compared. While HmbR-expressing cells used only hemoglobin (Hb) and heme, HemR-expressing bacteria were able to grow on Hb, heme, myoglobin, hemopexin, catalase, human and bovine serum albumin-heme, and haptoglobin-hemoglobin complexes as sources of iron. Expression of functional HemR allowed *Escherichia coli* cells to respond to heme-containing peptides, microperoxidases MP-8, MP-9, and MP-11, suggesting the ability of HemR to transport heme covalently linked to other molecules. Comparison of HemR with other heme receptors identified several highly conserved histidine residues as well as two conserved amino acid motifs, the FRAP and NPFL boxes. A site-directed mutagenesis approach was used to investigate the roles of His128, His192, His352, and His461 residues in HemR function. The HemR receptor with histidine changed to lysine at position 128 (HemR^{H128K}), HemR^{H461L}, HemR^{H461A}, and HemR^{H128A,H461A} mutant receptors were unable to use Hb, human serum albumin-heme, and myoglobin as sources of porphyrin and iron. Utilization of free heme was also severely affected, with some residual heme uptake in cells expressing HemR^{H128K}, HemR^{H461A}, and HemR^{H461L}. Conversely, the HemR^{H192T}, HemR^{H352A}, HemR^{H352K}, and HemR^{H192T,H352K} mutant receptors were fully functional. All mutant HemR proteins were expressed in the outer membrane at levels similar to that of the wild-type HemR receptor. Nonfunctional HemRs were able to bind heme- and Hb-agarose. A hypothetical model of the HemR function in which two conserved histidine residues, His128 and His461, participate in the transport of heme through the receptor pore is postulated.

The heme molecule (iron protoporphyrin IX) is one of the most important enzyme cofactors. Bacteria devote up to 10% of the total cell iron content and a significant amount of cell energy to the biosynthesis of heme (3, 33). The high energy cost of heme biosynthesis coupled with the constant famine that bacteria face in their natural environments favored the evolution of heme-scavenging systems. The heme-scavenging systems developed in different bacteria are confronted with the following tasks: (i) binding a heme-containing compound, (ii) releasing heme from the compound, and (iii) transporting heme into the cell. Studies with *Yersinia enterocolitica*, *Vibrio cholerae*, and *Haemophilus influenzae* have begun to define models of heme utilization in gram-negative bacteria (6, 13, 17, 43, 44). These studies postulated that all heme-utilizing bacteria possess outer membrane (OM) receptors that bind heme or heme-containing proteins and transport heme across the otherwise impermeable OM.

Bacterial receptors that interact with heme-protein complexes, transferrin (Tf), and lactoferrin (Lf) as sources of iron are close relatives of siderophore and vitamin B₁₂ receptors of gram-negative bacteria. However, while siderophore and vitamin B₁₂ receptors bind their cognate ligands directly, receptors that use heme-protein complexes, Tf, or Lf must first release the ligand (i.e., heme or iron in the case of Tf and Lf receptors) from the protein complex. This is not a trivial task, since the

bond between the heme and the globin in hemoglobin (Hb) has an association constant of 10¹² to 10¹⁶ (5). Similarly, the constant of association of Tf and iron is 10²⁰ (1). The mechanism and the protein domains involved in stripping heme or iron from protein complexes and transporting heme or iron through the receptor pore are currently unknown.

Heme and Hb are preferred substrates for the majority of bacterial heme acquisition systems (27). However, some bacteria use additional heme-protein complexes like hemopexin-heme, haptoglobin-Hb, heme-albumin, and myoglobin (8, 40, 48). *Serratia marcescens* responds to the challenge of multiple heme sources by producing a small protein, hemophore, that is able to extract heme from protein complexes and deliver it to the OM receptor (12, 28). *Vibrio vulnificus* and *Porphyromonas gingivalis* make heme available to their heme receptors by secreting proteases that degrade Hb and other heme-protein complexes (11, 38). Other heme-utilizing pathogens express multiple OM receptors that are specific for some but not all heme-protein complexes. *H. influenzae*, for example, expresses several distinct hemopexin and haptoglobin-hemoglobin utilization systems (6, 20, 32, 36). Conversely, when cloned into *Escherichia coli*, only one *Yersinia pestis* genetic locus allowed use of several heme-protein sources (18).

We have used the HemR protein of *Y. enterocolitica* as a model system to learn how heme and Hb receptors function. In this communication, we show that HemR of *Y. enterocolitica* is sufficient for the utilization of the largest variety of heme-containing compounds. HemR also used heme-peptide conjugates, which suggests that the receptor pore may accommodate molecules larger than heme itself. Amino acid comparisons of several heme and Hb receptors identified four conserved his-

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TABLE 1. Bacterial strains and plasmids used in the study

Bacterial strain or plasmid	Relevant genotype ^a	Source or reference
<i>Escherichia coli</i>		
DH5 α		Stratagene
IR1532	DH5 α but heme biosynthesis mutant	47
IR1583	As IR1532 but <i>tonB</i> <i>exbB</i> <i>exbD</i> ^{NM}	47
EB53	<i>E. coli</i> <i>hemA</i> <i>aroB</i> Rif ^r	43
IR754	EB53 but <i>tonB</i> ::Kan ^r	45
<i>Y. enterocolitica</i>		
WA-C		
H1883	Plasmidless strain, <i>hemPRSTVU</i> ⁺	43
H5030	<i>hemR</i> :: <i>phoA</i> Amp ^r	43
PLASMIDS		
pT76.91	pT7-5, <i>hemPR</i>	43
pHEM101	pBluescript <i>hemPR</i>	43
pT76.53	<i>hemPRST</i> ^r	43
pWKS130	Low-copy-number vector, Neo ^r	49
pWKS30	Low-copy-number vector, Amp ^r	49
pAMP1173	pWKS30 carrying <i>hmbR</i> ^{WT}	This study
pNEO76.91	pWKS130, <i>hemR</i> ^{WT}	Laboratory collection
pNEO1187	pWKS130, <i>hemR</i> ^{H128K}	This study
pNEO1188	pWKS130, <i>hemR</i> ^{H461K}	This study
pNEO1194	pWKS130, <i>hemR</i> ^{H352K}	This study
pWSH1332	pWKS130, <i>hemR</i> ^{H192T}	This study
pNEO1391	pWKS130, <i>hemR</i> ^{STOP}	This study
pICE1403	pWKS130, <i>hemR</i> ^{H192T,H352K}	This study
pNEO1505	pWKS130, <i>hemR</i> ^{H352A}	This study
pNEO1507	pWKS130, <i>hemR</i> ^{H461A}	This study
pCSB1707	pWKS130, <i>hemR</i> ^{H128A,H461A}	This study

^a *hemR*^{WT}, wild-type *hemR*.

tidine residues and a conserved receptor domain that was specific for heme and Hb receptors. Results from site-directed mutagenesis experiments confirmed that two of four conserved histidine residues in HemR are essential for its function.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study are listed in Table 1. Bacteria were routinely grown in Luria broth (LB). Iron-limiting conditions were accomplished by growing bacteria on nutrient broth plates supplemented with dipyrrolyl (NBD) (43). When necessary, supplementation with δ -aminolevulinic acid (final concentration, 50 μ g/ml) was used. Chemicals were purchased from Sigma (St. Louis, Mo.), Aldrich Co. (Milwaukee, Wis.), and Porphyrin Products, Inc. (Logan, Utah).

Growth promotion and inhibition assays. The functions of mutant HemRs were tested by assaying the abilities of these receptors to supply an *E. coli* heme biosynthesis mutant with heme-iron and/or heme-porphyrin while growing in the presence of different heme sources. Assays done on LB plates tested the ability of HemRs to supply heme as a porphyrin source to cells. Assays done on nutrient broth plates supplemented with 0.25 mM dipyrrolyl [NBD(0.25)] tested the ability of HemRs to supply heme as both an iron source and a porphyrin source. Testing of HemR function on LB plates allows detection of very small functional defects, since under these growth conditions, HemR is expressed at lower levels than when grown on NBD(0.25) plates (approximately 12-fold ratio of induction) (43). Human serum albumin (HSA), bovine serum albumin (BSA), horse heart muscle and skeletal muscle myoglobin, cytochrome *c*, catalase, and human Hb A₀ were purchased from Sigma and dissolved in 0.9% NaCl at a final concentration of 100 μ M (except for catalase [25 μ M]). One milligram of a lyophilized pooled human haptoglobin (Sigma) that binds between 0.5 and 0.9 mg of human Hb was dissolved in 100 μ l of a 6.5-mg/ml solution of human Hb. Ten microliters of the haptoglobin-Hb complex was used in growth promotion assays. Twenty microliters of human hemopexin (2.5 mg/ml), a gift from E. Hansen, was used in growth promotion assays. Hemin was dissolved in 20 mM NaOH (final concentration, 10 mM). Albumin-heme solutions were prepared by mixing heme and HSA (or BSA) in a 1:1 molar ratio. Approximately 2×10^7 *E. coli* IR1532 cells expressing the wild-type or mutant HemRs or *E. coli* 1583 cells expressing HmbR were inoculated into 3 ml of 0.75% agarose in water and spread onto LB or NBD plates. The HemR and HmbR receptors were expressed from their own promot-

ers. Paper discs (one to four discs, each 6 mm in diameter) were soaked with 10- μ l amounts of different heme compounds and placed on plates. Zones of stimulation were measured after 18 to 24 h of incubation at 37°C. The zones of growth stimulation obtained with *E. coli* IR1532(pNEO76.91) were very similar to the zones of growth stimulation seen with *E. coli* EB53(pNEO76.91), *E. coli* EB53(pT76.53), and *E. coli* IR1532(pT76.53) (data not shown).

Microperoxidases were purchased from Sigma and dissolved in 0.9% NaCl to a final concentration of 5 mg/ml. Discs with 10- μ l amounts of stock solutions were placed onto NBD(0.2) plates containing bacteria. δ -Aminolevulinic acid was added to the plates in experiments with *E. coli* EB53 to ensure bacterial growth. Due to the high bacterial inoculum and the presence of δ -aminolevulinic acid, *E. coli* EB53 could grow on NBD(0.2) (background growth visible after 1 to 2 days of incubation).

Site-directed mutagenesis of *hemR*. HemR mutants were constructed by using either PCR amplification or the QuickChange protocol (Stratagene). Plasmid pHEM101, which had been linearized with *ScaI*, was used as a template for mutagenesis. A unique restriction site (*HindIII* or *SpeI*) was introduced at the locations of His codons by PCR amplification with the *PwoI* DNA polymerase (Boehringer, Mannheim, Germany). His128 was replaced by a lysine residue, creating a unique *HindIII* restriction site. His192 was replaced by a threonine residue, creating a new *SpeI* restriction site. His352 was replaced by a lysine residue, creating a new *HindIII* restriction site. His461 was replaced by a leucine residue, creating a new *HindIII* restriction site. Other mutant HemRs (HemR receptor with H changed to A at position 352 [HemR^{H352A}], HemR^{H461A}, and HemR^{H128A,H461A}) were constructed by the QuickChange protocol (Stratagene) with pT76.91 as a template for mutagenesis. The *hemR*^{H128A} mutation can be detected by *NarI* digestion. Cartridge-purified PCR primers, 33 to 36 bp in length, were purchased from Gibco BRL. DNA fragments carrying the mutations (*HpaI-KpnI*, *SacII-KpnI*, or *EcoRV-EcoRV* [see Fig. 4]) were subcloned into pBluescript and recloned into the wild-type pT76.91 (43). All DNA fragments carrying the mutations were sequenced to rule out secondary mutations. Finally, the *hemR* gene, expressed from its own promoter, was subcloned onto a low-copy-number vector pWKS130 in the orientation opposite that of the *lac* promoter (49). The amount of HemR expressed from the low-copy-number plasmid approximated the amount of the receptor expressed in *Y. enterocolitica* cells (43).

OM fraction preparation. Triton X-100 OM fractions were prepared from *E. coli* IR1532, a DH5 α heme biosynthesis mutant, expressing recombinant HemRs from low-copy-number (pWKS130) and medium-copy-number (pT7-5) plasmids. Briefly, 100-ml cultures were inoculated with 1-ml portions of cultures grown overnight and incubated at 37°C with shaking for 2.5 h. An iron chelator, dipyrrolyl (final concentration 0.3 mM) was added to induce the expression of HemRs, and the cultures were incubated for a further 2.5 h. Cells were harvested by centrifugation and resuspended in 1 ml of 200 mM Tris (pH 8.0), followed by addition of 2 ml of 1 M sucrose in 200 mM Tris (pH 8.0)–200 μ l of 10 mM EDTA–200 μ l of lysozyme at 2 mg/ml–6.4 ml of double-distilled water (ddH₂O). Mixtures were left to stand at room temperature for 5 min to allow spheroplasts to form. Ten milliliters of a solution of 2% Triton X-100, 50 mM Tris (pH 8.0), 10 mM MgCl₂, and 200 μ l of DNase at 1 mg/ml was added, and the mixtures were spun at 18,000 rpm in a JA25.50 rotor for 60 min at 4°C. Triton X-100-insoluble OM fraction pellets were washed three times in 1 ml of ddH₂O and resuspended in ddH₂O to a final volume of 100 μ l (14).

Affinity chromatography of HemR with heme and Hb-agarose. OM preparations from bacteria expressing HemRs from medium-copy-number plasmids (pT7-5) were solubilized for affinity chromatography in Zwittergent 3-14 (9, 27). OM samples were diluted in 600 μ l of phosphate-buffered saline (PBS) and then 600 μ l of 1% Zwittergent 3-14 in PBS was added. Binding reactions were performed in Eppendorf tubes with 10 to 20 μ l of hemin-agarose (Sigma H6390; 7.7 μ mol of hemin per ml of packed gel) or 20 μ l of Hb-agarose (Sigma H8756; 16.7 mg of Hb per ml of packed gel). Binding reaction mixtures were incubated on a rotator either for 2 h at room temperature or overnight at 4°C. Agarose was then washed four times with 1.2 ml of 0.5% Zwittergent 3-14 in PBS to remove unbound material. Agarose was boiled in Laemmli sample buffer for 3 min and then electrophoresed in a 10% polyacrylamide gel at constant voltage with Tris-glycine buffer (24). Proteins in the gel were visualized with Coomassie stain.

RESULTS

The HemR receptor uses a wide spectrum of heme compounds. Use of different heme-containing compounds by an *E. coli* heme biosynthesis mutant expressing the *Y. enterocolitica* HemR and *N. meningitidis* HmbR was compared (45, 46). As can be seen from Fig. 1, the HemR-expressing strain was able to use heme, heart and skeletal muscle myoglobins (MY-M and MY-H), Hb, BSA- and HSA-heme complexes, hemopexin, and catalase as the sole sources of porphyrin and iron. The use of haptoglobin-Hb complexes (HPT-Hb) was also dependent on the expression of HemR, while both native and denatured cytochrome *c* could not support growth of HemR-expressing cells. Conversely, the strain expressing the HmbR receptor

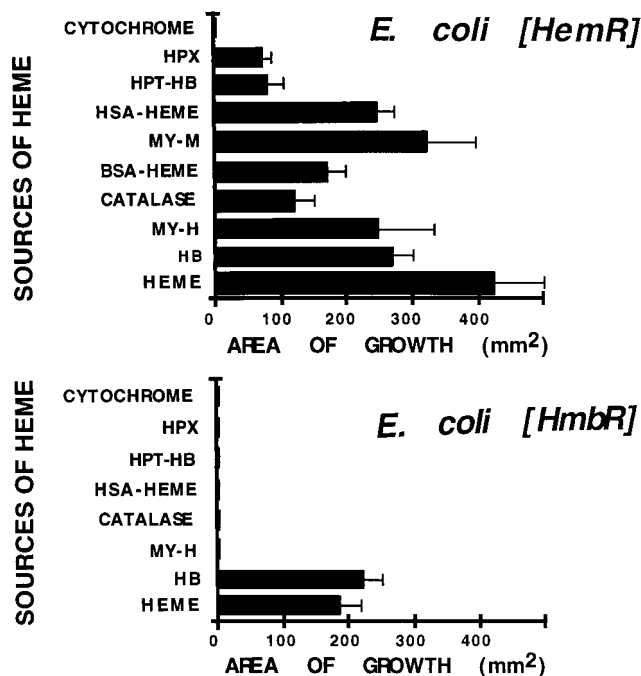


FIG. 1. Comparison of the abilities of heme and Hb receptors, HemR and HmbR, to use different heme-protein complexes as sources of iron and porphyrin in the *E. coli* heme biosynthesis mutant IR1532. HemR and HmbR receptors were expressed from low-copy-number plasmids pWKS130 and pWKS30, respectively (49). The expression of HmbR was done in the presence of *N. meningitidis* *tonB* *exxB* *exxD* genes (47). Bars represent the surface areas of bacterial growth around the discs on NBD plates (error bars show the standard deviations). Discs were soaked with equimolar amounts of heme-containing compounds (except for heme, see Material and Methods). Each measurement was repeated at least three times. Abbreviations: HB, hemoglobin; HPX, human hemopexin; HPT-HB, human haptoglobin-Hb complex; MY-M, skeletal muscle myoglobin; MY-H, heart muscle myoglobin.

used only heme and Hb as sources of porphyrin and iron (Fig. 1). The spectrum of heme-containing compounds used by these receptors correlated with the spectrum of heme compounds used by yersiniae and neisseriae (8, 18, 40, 43, 45). The inability of HmbR-expressing cells to use some heme-protein complexes ruled out the possibility that heme released from denatured complexes fed HemR-expressing bacteria. These results show that the expression of the HemR receptor is sufficient for utilization of heme from native heme-protein complexes.

The HemR receptor-expressing bacteria use a heme-peptide conjugate as a source of iron. The inability of the native and denatured cytochrome *c* to serve as an iron source indicated that covalently attached heme cannot be taken up by the HemR receptor (Fig. 2). Interestingly, peptides derived from cytochrome *c* by proteolytic digestion, such as microperoxidases MP-8 and MP-9, stimulated growth, whereas MP-11 inhibited growth of *Y. enterocolitica* under iron-limiting growth conditions (Fig. 2). The *hemR* mutant of *Y. enterocolitica* was not stimulated by MP-8 and MP-9 or inhibited by MP-11. All three compounds were inhibitory for HemR-expressing *E. coli*. The heme moiety is covalently attached to a peptide in MP-11, MP-9, and MP-8, and these compounds have peroxidase activity (15). *E. coli* cells expressing the *N. meningitidis* HmbR protein were not sensitive to MP-11. The toxicity of microperoxidases depended on the genotype of the strain and growth conditions used in the experiments. Only HemR-expressing *E. coli* strains that possessed functional TonB protein were sen-

sitive to these compounds. The presence of heme and Hb partially protected sensitive, HemR-expressing *E. coli* cells against MP-11 toxicity (data not shown). These results suggest that the HemR receptor recognizes the heme moiety of MP-11 and transports the whole compound into the bacterial periplasm. The mechanism by which microperoxidases inhibit bacterial growth and the reason for a difference in sensitivity of *E. coli* and *Y. enterocolitica* to MP-8 and MP-9 are not clear.

Identification of conserved amino acid motifs present in different heme and Hb receptors. The mechanism and protein domains by which heme receptors bind heme compounds, extract heme from these compounds, and transport heme into the cell are not known. Amino acid comparisons of different TonB-dependent siderophore receptors identified several conserved domains and amino acid residues (21). Similar analysis of bacterial heme receptors revealed two amino acid motifs that are highly conserved among different heme and Hb receptors but are not present or conserved in siderophore and vitamin B₁₂ receptors (Fig. 3A). The FRAP and NPPL amino acid motifs and a conserved His residue between the motifs were found only in heme receptors and were not well conserved in siderophore and vitamin B₁₂ receptors. The FRAP box and the His residue at position 461 were also present in some Tf and Lf receptors. The same analysis revealed that several heme receptors possess additional conserved histidine residues, corresponding to His128, His192, and His352 of HemR (Fig. 3B). Histidine 128 is located in the receptor domain that is conserved in all TonB-dependent receptors (21, 31). However, His residues were not found at the corresponding regions of FepA and FhuA receptors. The *E. coli* FepA receptor contains histidine residues at positions 194, 351, 473, and 474, but there is no significant homology between other parts of these histidine-containing domains (data not shown).

Comparison of the HemR model with crystal structures of FepA and FhuA. The overall homology of *E. coli* FhuA and FepA and *Y. enterocolitica* HemR is relatively low: HemR shares 21% identical amino acid residues with FhuA and FepA. A two-dimensional model of the HemR receptor was designed in order to determine probable localization of the FRAP and NPPL boxes and conserved histidine residues on the receptor (42). Amino acid comparison analysis of HemR and its close relatives, heme receptors ChuA, SduA, HmuR, and PhuR, was used to assist in the determination of surface-exposed loops and transmembrane regions of the receptor. The model of the HemR receptor contains 22 transmembrane β -strands and 11 surface-exposed loops (data not shown). The first ~150 amino acid residues together with the TonB box are localized in the periplasm, closing the receptor pore. This arrangement of the amino terminus of the receptor is based on the three-dimensional structures of FepA and FhuA receptors (4, 10, 30). The amino-terminal domain of HemR contains the invariable His128 residue. His128 aligns with arginine 105 of FepA and tyrosine 116 of FhuA. Arginine residues of the FepA amino terminus have been postulated to interact with the negatively charged enterobactin sideropore (4), while tyrosine 116 was found to make hydrogen bonds with the ferrichrome (10, 30). The HemR model predicted that the FRAP and NPPL boxes and His352 are localized in the transmembrane regions, while His192 and His461 are localized in the surface-exposed loops of the receptor. A limited homology between the FepA domain encompassing residues 485 to 532 and the HemR domain containing FRAP and NPPL motifs was found (Fig. 3A). Corresponding FepA residues participate in the formation of two β -sheets and an external loop, suggesting that the placement of FRAP and NPPL motifs is correct in the two-dimensional model of HemR (i.e., that the loop containing an

STRAINS	HEME COMPOUNDS				
	MP-8	MP-9	MP-11	cyt. <i>c</i>	Heme
<i>E. coli</i> <i>wt</i>	R	R	R	-	-
<i>E. coli</i> <i>wt</i> / <i>hemPRST'</i>	(S, 22)	(S, 22)	(S, 23)	-	+
<i>E. coli tonB::Kan</i> / <i>hemPRST'</i>	R	R	R	-	-
<i>E. coli</i> -1532 / <i>hmb R</i>	ND	ND	R	-	+
<i>Y. enterocolitica</i> <i>hemR</i> ⁺	+	+	(S, 22)	-	+
<i>Y. enterocolitica</i> <i>hemR</i> ⁻	-	-	R	-	-

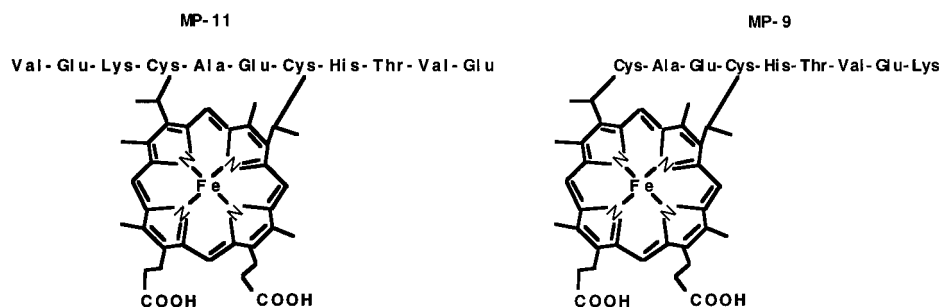


FIG. 2. Growth-inhibitory or -stimulatory activity of microperoxidases on *Y. enterocolitica* and *E. coli* (top) and structures of microperoxidases MP-9 and MP-11 (bottom). Wild-type *E. coli hemA* *aroB* (*E. coli*^{wt}) and *E. coli* IR1532 (*E. coli*-1532), a DH5 α heme biosynthesis mutant (47), were two of the strains used. Growth-inhibitory or -stimulatory activity of microperoxidases MP-8, MP-9, and MP-11, cytochrome *c* (cyt. *c*), and heme are shown as follows: R, resistant to the inhibitory activity of microperoxidases; (S, 22), sensitive to inhibitory activity of microperoxidases, with a 22-mm-diameter inhibition zone; ND, not done; +, stimulation of growth under iron-limiting conditions; -, no stimulation of growth under iron-limiting conditions. Only bacteria that possess functional HemR can be stimulated or inhibited by microperoxidases. The same results were obtained with the native and denatured forms of cytochrome *c*. MP-8 is one amino acid (Lys) shorter than MP-9 (not shown). Note a covalent link between the peptide and the heme moiety.

invariant His461 is exposed on the surface). This prediction could not be independently tested with the FhuA model due to the lack of homology between HemR and FhuA receptors at corresponding regions.

Mutagenesis and phenotypic characterization of the HemR histidine mutants. Conserved histidine residues of HemR may play an important role in binding of heme-protein complexes and/or transporting heme through the receptor pore. The role of conserved His residues in HemR function was tested by constructing site-directed HemR mutants (Fig. 4). Mutant receptor genes were expressed from the *hemR* iron-regulated promoter (43). All mutant receptors localized to the OM in approximately equal amounts (Fig. 5 and data not shown). The ability of the *E. coli* IR1532 heme biosynthesis mutant (47), transformed with different HemRs, to use 100 μ M solutions of Hb, myoglobin, HSA-heme complexes, and heme (10 mM) as sources of heme and iron was assessed by the plate assay (see below). Plate assays were done on both NBD and LB plates in order to test the abilities of HemRs to use heme-protein complexes as sources of porphyrin and iron and porphyrin alone. In addition, testing mutants under iron-rich (LB plates) and iron-poor (NBD plates) growth conditions allows comparison of the receptor function at different levels of expression, since the expression of the *hemR* gene is regulated by the availability of iron (approximately 12-fold) (43). The mutant recep-

tors HemR^{H128K} (pNEO1187), HemR^{H461L} (pNEO1188), HemR^{H461A} (pNEO1507), and HemR^{H128A,H461A} (pCSB1707) could not support growth of the *E. coli* heme biosynthesis mutant on any heme source when tested on LB plates (Fig. 4A). The HemR receptors HemR^{H192T} (pWSH1332), HemR^{H352K} (pNEO1194), HemR^{H352A} (pNEO1505), and HemR^{H352K,H192T} (pICE1403) were proficient in utilization of all heme sources on LB plates (Fig. 4A).

Testing the mutant HemRs under iron-restricted growth conditions gave the following results: HemR^{H461L} and HemR^{H461A} were unable to use Hb, myoglobin, and HSA-heme, while free heme still had some growth-promoting effect. The *hemR*^{H461A} allele had a much larger growth defect on heme than the *hemR*^{H461L} allele. The *hemR*^{H128A,H461A} double mutant was the most affected receptor allele; only a 1-mm-wide and weak growth zone could be seen around the heme disc (Fig. 4). HemR^{H128K} was grossly affected in its ability to use three heme-protein complexes; however, a very small and weak zone of growth around the Hb disc was observed on the lawn of *E. coli* 1532(pNEO1187). The zone of growth around the heme disc was significantly smaller than the one observed with the wild-type HemR but larger than the zone of growth observed with the HemR^{H461L} receptor (Fig. 4). Cells expressing the HemR^{H352K} receptor grew slower and needed longer to form growth zones around different heme-containing discs,

A	siderophore receptors	{	IUTA	401...TG- R YPSYDITNLAAPLQSGYDI--	NNLFTLNGGVRYQ
			FHUE	636...TS Y RLPVMPELTVGGGVNWQNRVY--	TDVTVPYGTFR A E
			FOXA	547...-AN R AVPATY-YVPAGKVNSQGL---	ELEARSQISDR L S
			FEPa	464...-A Y KAPSL---YQTNPNYILYSKGQ-	NDDLKA E T S IN K E
			CIRA	418...TA F KAPSLQLSPDWTNSCRGACKI	SPDL K P E T S ES W E
	vitaminB12 receptor	{	BTUB	401...EG Y RFIASYGT S YKAPNLGQLYGYG	NP N L N PE K SK Q W E
	Iron (III) receptors	{	HITBP1	642...NA F R A P T SDEI Y MT F K H P D F S IG P --	NT N L K A E T A K T K E
			TBPB16	639...TG F R L P S F A EMWELGLAGSGKAG N FK	LS G F K T K Y R D F I E
			LPB	670...SG F R T P S F Q EL F G I D I Y H D Y PK G W Q --	RP A L K S E K A AN R E
	heme, Hb, and Hb-Hpt receptors	{	HEMR	445...QA F R A P T MG E MY N DS K H P DF Y L K --A	NP N L K A E K A K N W E
			HGBA	690...TG F R A P T SDEI Y F V F Q H P S F S I Y--P	N L Y L K A E R S K N K E
			HMUR	445...QA F R A P T MG E MY N DS K H F A I P I R P --	NP N L K P E T N E T Q E
			HPUB	534...TG F R A P T SDE T W L L F P H P D F Y L K --A	NP N L K A E K A K N W E
			CHUA	432...QA F R A P T MG E MY N DS K H F S I G R F Y T N	NP N L R P E T N E T Q E
HMBRC			508...S G Y R V P N A SEV Y F T Y N H G S G N W L--P	NP N L K A E R T T H T	
HXUC			467...E A F R A P S M Q E R F V S G S H F G T S I L G R N	NP N L R P E T A K N K E	
HASR			620...K S W R P P A I T E T L T N G S A H S S T Q Y P	NP F L Q P E R S R A W E	
HI0635			643...NA F R A P T SDEI Y MT F K H P D F S IG P .	NT N L K A E T A K T K E	
HUTA			458...Q G F R A P D F Q E L Y S F G N P A H G Y V F K P	NP N L E A E D S V S Y E	
HMBRA			508...S G Y R V P N A SEV Y F T Y N H G S G N W L---P	NP N L K A E R S T T H T	
SHUA			432...QA F R A P T MG E MY N DS K H F S I G R F Y T N	NP N L R P E T N E T Q E	
HMBRB			508...S G F R V P N A SEV Y F T Y N H G S G T W K---P	NP N L K A E R S T T H T	
HI0661			489...K G F R T P T S D E M Y T F K H P D F T I L P..	NT D L K P E I A K T K E	
HHUA			761...-- F R A P T SDEI Y F T F L H P D F S I R P --	N L D R Q A E T A K T K E	
HI0712	770...N G F R A P T S D E I Y M T F K H P K F S I Q P ..	NT D L K A E T S K T K E			
	**** * Aro *	**** ** *			

B	HIS 128			
	FEPa	91	--SRNSVRQWRGERD R GT S WV P PE M I E	123
	FHuA	99	GKSQNNYLNGLKLQGN F Y N DA V --ID P Y M L E	127
	HEMR	112	GVLTLVDGIRQ G T D -T G H L N S T F L D P A L V K	140
	HMUR	112	GVLTLVDGVRQ G T D -T G H L N S T F L D P A L V K	130
	CHUA	98	GVLVLVDGVRQ G T D -T G H L N G T F L D P A L I K	126
	SDUA	98	GVLVLVDGIRQ G T D -T G H L N G T F L D P A L I K	126
	HXUC	92	R V V Q V I D G V R Q N F D -L A H R G S Y F L P M S L I Q	120
	HASR	204	R V N M N I D G M R Q N F Q K S G H G Q R N G T M I D S E	234
			* . ** . * . *	
	HIS 192			
	HEMR	172	LLLPQNSGYRVYSSAA-TGD H S F GLGASAFGR-T	204
	HMUR	172	MLQPQNSGYRVYSSAA-TGD H S F GLGASAFGR-T	204
	CHUA	158	LLQEQSSGFVFGT G G-TGD H S L GLGASAFGR-T	190
	SDUA	158	LLQEQSSGFVFGT G G-TGD H S L GLGASAFGR-T	190
	HASR	270	FLAPGKELGGKLHASTGD N G T H F IGSGILALGN E T	304
			* * * . . * * . * * * *	
	HIS 352			
	HEMR	341	R T R L F T D S F A S H L L T Y G T E A Y K	362
	HMUR	341	R T R L F I E S P A S H L L T Y G T E T Y K	362
	CHUA	328	R S T L F A D S F A S H L L T Y G G E Y Y R	349
	SDUA	328	R S T L F A D S F A S H L L T Y G G E Y Y R	349
	HMBRC	371	R F K R I T L R L D S H P L Q L G G R H R	392
			* . . * * * . *	

FIG. 3. Amino acid comparisons of the conserved domains of the heme and Hb receptors. (A) A highly conserved receptor domain containing an invariant histidine residue, FRAP and NP**N**L amino acid boxes, present in all receptors that transport heme into the periplasm. Siderophore, vitamin B₁₂, and some Tf and Lf receptors lack either the complete domain or have only the FRAP box and distal glutamic amino acid residues relatively well conserved. Highly conserved residues (indicated by asterisks), aromatic residue (Aro), and gaps introduced to maximize alignment (indicated by dashes) are shown. Hpt, haptoglobin. (B) Amino acid comparison of Hb and heme receptor domains around conserved histidine residues (conserved histidine residues are shown in bold). *Y. enterocolitica* HemR (HEMR), *Y. pestis* HmuR (HMUR), *E. coli* ChuA (CHUA), *S. dysenteriae* SduA (SHUA), *H. influenzae* HxuC (HXUC), *E. coli* FepA (FEPa), and *E. coli* FhuA (FHUA) are shown. Amino acid residues of all receptors except FepA and FhuA are numbered from the first methionine.

although the diameters of the zones were identical to the ones seen with the wild-type cells. The growth of cells expressing HemR^{H352A} and the wild-type receptor were indistinguishable. Growth zones of HemR^{H192T}-expressing cells around myoglobin and heme and were larger than those found in the wild-type strain (Fig. 4A).

Quantification of the defects of individual HemR mutant receptors. The growth defects of the mutant HemRs were quantified by expressing the mutant genes in *E. coli hemA aroB* (EB53) cells and measuring the kinetics of growth in liquid medium in the presence of different heme compounds. The

best sources of porphyrin for the wild-type HemR-expressing bacteria were Hb and heme, allowing growth at a concentration of 1 μM. Fifty- to 75-fold higher concentrations of myoglobin were needed to obtain the same kinetics of growth (data not shown). There was a clear difference in the ability of the HemR-expressing cells to use HSA- and BSA-heme complexes; 50-fold higher concentrations of HSA-heme were needed to obtain the growth observed with BSA-heme, Hb, or heme (Fig. 6).

Strains expressing HemR^{H128K} and HemR^{H461L} could not grow in the presence of any of the heme compounds (Fig. 6 and data not shown). The phenotypes of the wild-type receptor and HemR^{H192T} were identical, irrespective of the heme source, as were the phenotypes of receptors HemR^{H352A} and HemR^{H192T.H352K} (Fig. 6 and data not shown). The HemR^{H352K}-expressing cells grew slower than the wild-type cells in the presence of 1 μM Hb, 50 μM myoglobin, and 1 μM heme; however, no difference in growth was observed with HSA-heme and BSA-heme as the sources of heme (Fig. 6).

Binding of mutant HemRs to heme- and Hb-agarose. The abilities of HemR^{H461L}, HemR^{H128K}, and the wild-type receptor to bind heme and Hb were assessed by affinity purification of HemRs from bacterial OMs with heme- and Hb-agarose (9, 26). The wild-type receptor and the HemR^{H461L}, HemR^{H128K}, HemR^{H352K}, HemR^{H461A}, and HemR^{H128A.H461A} mutant re-

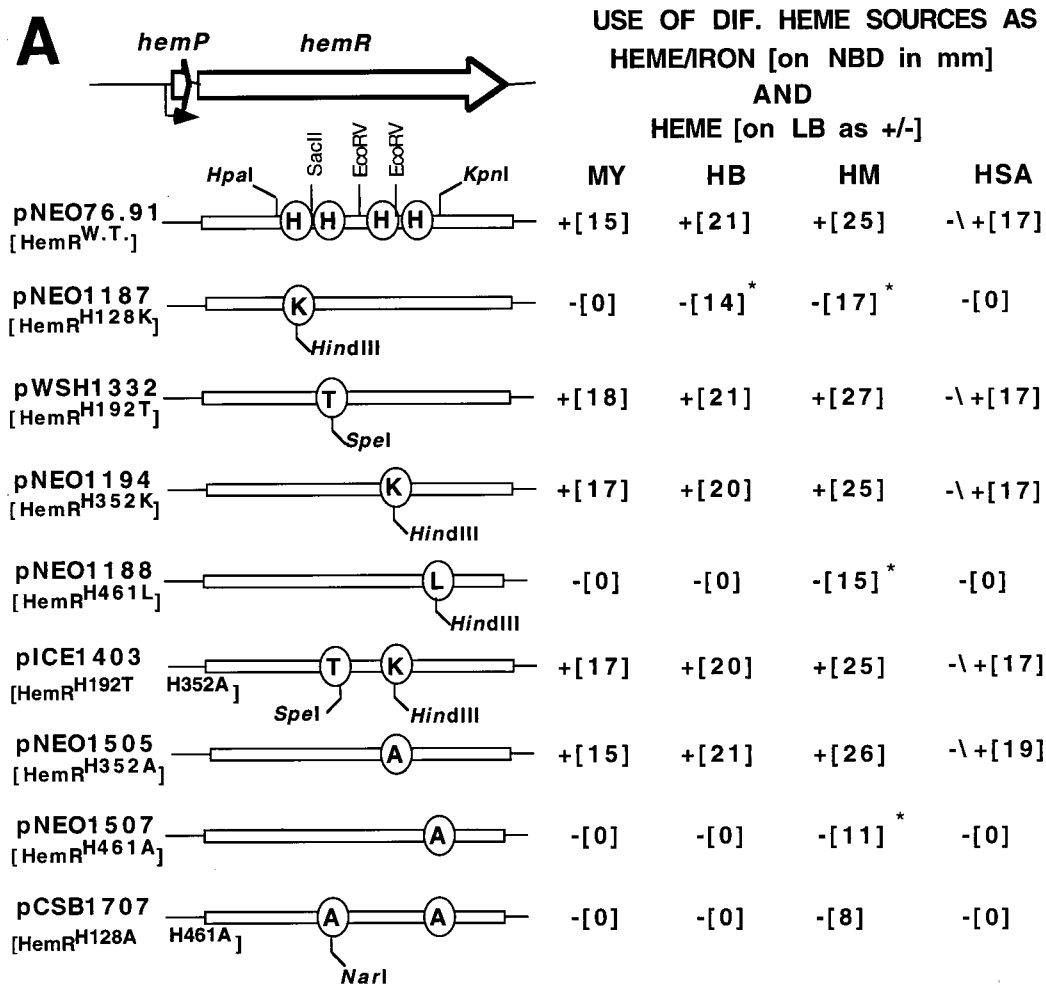


FIG. 4. (A) A schematic representation of mutant HemRs and their phenotypes after expression in the *E. coli* heme biosynthesis mutant. The testing for growth stimulation was done on LB and NBD plates essentially as described in the legend to Fig. 1. Growth stimulation with four different (DIF.) heme sources, myoglobin (MY), hemoglobin (HB), heme (HM), and HSA (HSA-heme complex, is indicated as follows: +, stimulation of growth; -, no stimulation; * and -/+, very light or small zone of stimulation. HemR^{W.T.}, wild-type HemR. (B) Growth zones of HemR histidine mutants around discs soaked in 10 μ l of 10 mM heme (NBD plates). WT, wild type.

ceptors were able to bind to heme- and Hb-agarose in very similar amount (Fig. 7 and data not shown). These data suggest that His128 and His461 are not the receptor residues that mediate binding to Hb and heme. Alternatively, HemR possesses several Hb and/or heme binding sites on its surface and the removal of only one site did not grossly affect the ability of the mutant HemR to bind to Hb- and heme-agarose.

DISCUSSION

A very large number of microorganisms express heme and Hb receptors which allow bacteria to use heme and heme-protein complexes as the sources of iron and porphyrin (27). Our understanding of the mechanism of action of these proteins and their interactions with different heme-containing compounds is very limited. We show that the expression of the *Y. enterocolitica* HemR OM receptor enables *E. coli* to use Hb, myoglobin, heme, HSA-heme, and BSA-heme as sources of iron and porphyrin. The HemR-expressing bacteria also used catalase, haptoglobin-Hb complexes, and hemopexin but were not able to use cytochrome *c*. Different heme compounds were not used with equal efficiency; 50- to 75-fold-higher levels of

myoglobin and HSA over those of Hb, heme, and BSA complexes were required in order to accomplish the same growth stimulation. These results are expected since human myoglobin binds heme with a higher affinity than Hb, and the affinity of human albumin for heme is 1,000 times higher than the affinity of heme for bovine albumin (5, 7).

The results of the assays using microperoxidases implicated the HemR receptor in the transport of heme-peptide conjugates across the OM. Although the transport of heme-peptides has not been demonstrated, dependence of microperoxidase-associated phenotypes on functional TonB and HemR proteins suggests an active transport process across the membrane. HemR is the first bacterial heme receptor that uses such a broad spectrum of heme-containing compounds. Indeed, the phenotypic analysis of another heme or Hb receptor, the HmbR protein of *N. meningitidis*, showed a very limited spectrum of usable heme sources. These phenotypic differences between the two heme and Hb receptors are in accordance with the relatively moderate level of similarity between their amino acid sequences. Conversely, a very high level of similarity (>65% identity) between HemR and its homologues found

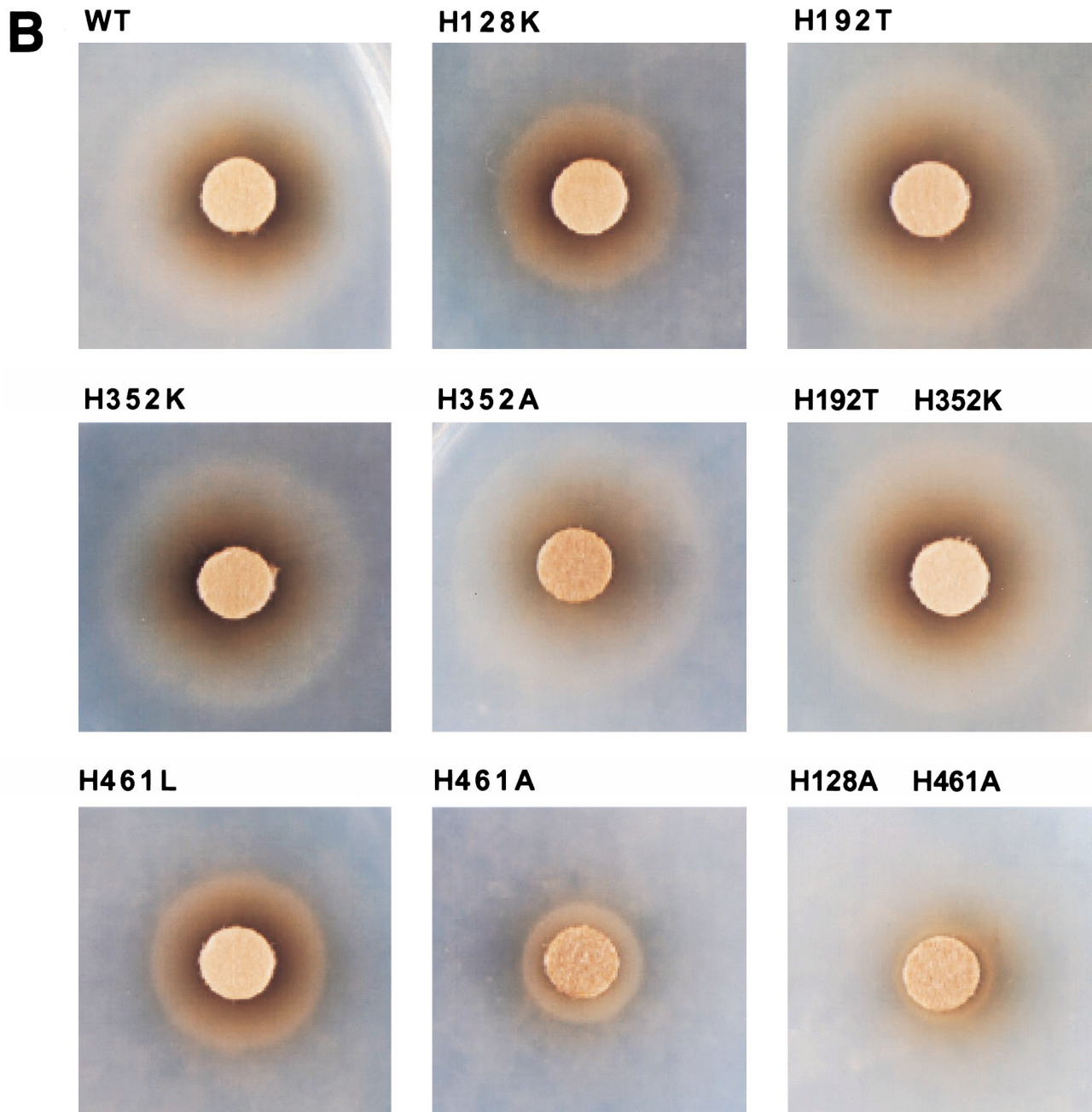


FIG. 4—Continued.

in *Shigella dysenteriae*, enterohemorrhagic *E. coli*, and *Y. pestis* suggests that these receptors may also allow use of a wide variety of heme-protein sources. Therefore, both amino acid comparisons and a limited phenotypic analysis indicate the existence of at least two families of heme and Hb receptors: receptors able to use a wide variety of heme sources (heme scavengers) and receptors restricted to one or two heme sources.

What is the significance of these findings for organisms that express heme and Hb receptors? It can be argued that the ability to scavenge heme from a variety of protein complexes may be advantageous to yersiniae and other members of the family *Enterobacteriaceae* that colonize several distinct ecolog-

ical niches. Heme receptors that are less finicky may also allow microorganisms to use and/or recycle heme derivatives such as the following: heme *o* (2-farnesylethyl heme *b*) found in bacterial cytochrome complexes; heme *a* (8-formyl heme *o*) found in terminal oxidase in animal cells and bacteria; heme *d* (5,6-bishydroxy heme *b*) found in bacterial cytochrome and *E. coli* catalase; heme *d*₁ which is widely present in denitrifying bacteria; and siroheme (heme *b* with saturated porphyrin ring), a prosthetic group of nitrite and sulfite reductases (3). Recycling of these valuable cofactors may have been the first metabolic role of heme receptors. On the other hand, highly host-adapted pathogens like *Haemophilus* and neisseriae, “specialized” their heme and Hb receptors to a particular heme source

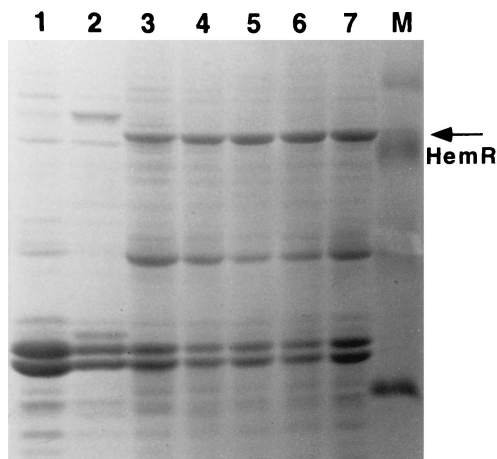


FIG. 5. OMs of *E. coli* expressing different HemR histidine mutants from a pWKS130 plasmid. Lanes: 1, HemR^{STOP} (pNEO1391 [see Table 1]); 2, *E. coli* DH5 α ; 3, HemR^{H352K}; 4, HemR^{H461L}; 5, HemR^{H192T}; 6, HemR^{H128K}; 7, wild-type HemR; M, molecular mass markers (30, 46, 69, and 94 kDa).

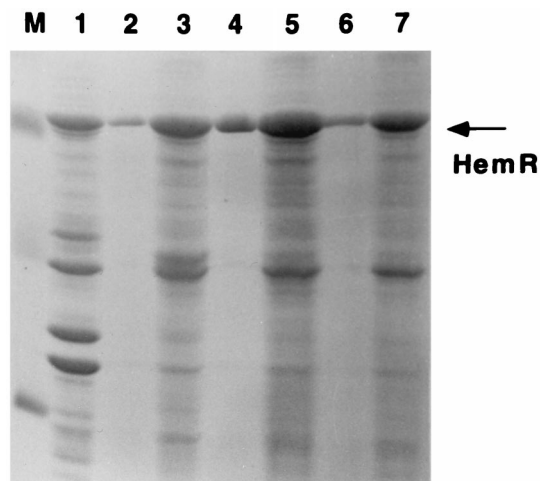


FIG. 7. Binding of mutant HemRs and wild-type HemR (HemR^{WT}) to heme- and Hb-agarose. Lanes: M, molecular size markers; 1, OMs of *E. coli* expressing HemR^{WT}; 2, Hb-agarose-bound HemR^{WT}; 3, heme-agarose-bound HemR^{WT}; 4, Hb-agarose-bound HemR^{H128K}; 5, heme-agarose-bound HemR^{H128K}; 6, Hb-agarose-bound HemR^{H461L}; 7, heme-agarose-bound HemR^{H461L}. Differences in the amount of material bound to heme and Hb-agarose are due to the 30-fold-higher binding capacity of the heme- over Hb-agarose.

that is encountered in the host. These data should not be interpreted as indicating that the HemR design is superior to the HmbR design. Quite to the contrary, HmbR-expressing bacteria may be more efficient than HemR-expressing bacteria when confronted with Hb as the sole heme substrate. On the other hand, the design of HemR probably did not need any fine adjustments to fit a specific heme-protein complex because yersiniae satisfy their iron needs by producing a very powerful siderophore (16).

What is the mechanism of HemR's function and how does HemR interact with different heme-protein complexes? The HemR receptor must be able to bind heme-protein complexes on its surface. The receptor either possesses several discrete binding sites for each heme-protein complex or has only one site that recognizes a common motif in all heme-protein complexes. After initial binding of a heme-protein complex, the

receptor must release heme from the complex and transport heme into the bacterial periplasm. Both steps should involve receptor residues that specifically recognize the heme molecule. Histidines are common axial ligands of heme-iron in proteins, and it is likely that these residues play an important role in HemR function (7, 39). Indeed, site-directed mutagenesis of conserved His residues of HemR confirmed the involvement of His128 and His461 in heme utilization. The HemR His461 mutants were more severely affected in function than the HemR^{H128K} receptor which retained some residual activity. Different phenotypes of these *hemR* alleles may be caused by a particular amino acid substitution. Alternatively, these

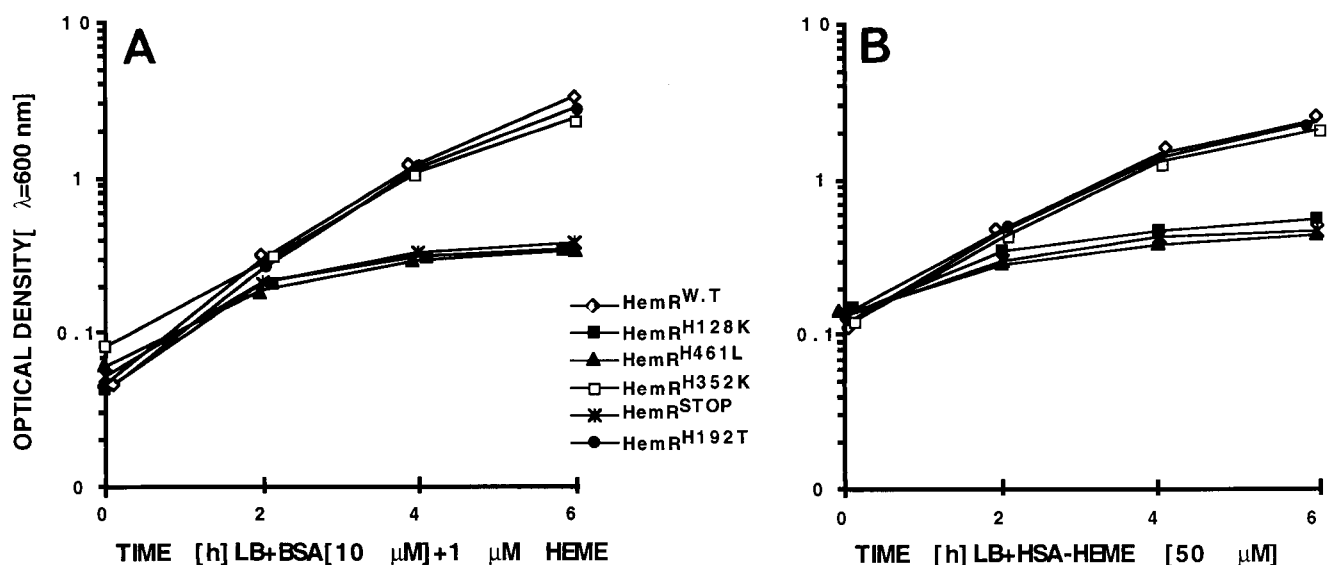


FIG. 6. Ability of *E. coli* EB53 (*hemA aroB*) cells expressing HemR mutant receptors to use BSA-heme complexes (10 μ M BSA and 1 μ M heme) (A) and HSA-heme complexes (50 μ M heme) as sources of heme. HemR^{W.T}, wild-type HemR; HemR^{STOP}, pNEO1391. Two to three independent growth measurements were conducted, producing essentially identical results.

results may suggest that the His128 and His461 function at different steps of the heme utilization process. However, these functionally important histidine residues are not essential for Hb binding, since HemR^{H128K}, HemR^{H461L}, HemR^{H461A}, and HemR^{H128A,H461A} mutants were still able to bind to Hb-agarose.

The His128 residue is localized in the part of HemR that is conserved among all TonB-dependent receptors (21, 31). The same region of FepA was shown to be essential for enterobactin uptake by both deletion and linker insertion analyses (2, 37). Similar analysis of homologous segments of the FhuA and BtuB receptors confirmed the importance of the amino-terminal region in receptor function (22, 23, 25). Recent crystallization studies of FhuA and FepA receptors revealed the role of the amino-terminal cork-like domain in receptor function: these domains close the receptor pore from the periplasmic side and participate in the formation of a substrate binding site (4, 10, 30). The location of His128 suggests that this residue participates in the formation of the heme binding site. Indeed, His128 aligns very well with Tyr116 of FhuA (Fig. 3B) which forms one of the apices of the amino-terminal cork domain and participates in the formation of the ferrichrome binding pocket (10, 30).

Comparison of HemR with siderophore receptors showed that the domain containing the histidine residue 461 is not well conserved in siderophore receptors but extremely well conserved among heme and Hb receptors. The whole domain is most likely specific for heme and Hb receptors. Since both the use of free and complexed heme is grossly affected in strains expressing HemR^{H461L} and HemR^{H461A}, His461 is probably a part of a surface-exposed, high-affinity heme binding site. This residue may participate in removal of heme from different protein complexes and in "catching" free heme molecules on the receptor surface. The ability of the HemR^{H461L} mutant to bind heme agarose does not contradict the role of His461 in heme binding. As suggested for the ferrichrome receptor, HemR most likely possesses several weak ligand binding sites on its surface (30). These sites "concentrate" heme close to His461, improving the efficacy of the receptor. Low-affinity heme binding sites might be responsible for interactions between HemR and heme-agarose.

We postulate that two HemR histidine residues, H128 and H461, interact with the heme molecule which is reminiscent of myoglobin and Hb heme pockets where two histidines hold heme through interaction with the central iron atom (7). However, considering that the function of HemR is not to hold heme but to transport it into the cell, it seems more feasible that the heme molecule is bound to only one histidine residue at a time, thus making the dissociation of heme from the receptor more favorable (41). Conformational changes that occur upon ligand binding and after recognition of the loaded receptor by TonB (19, 24a, 29, 34, 35) would be responsible for altering the affinities of H128 and H461 in such a way as to allow transfer of heme first from H461 to H128 and then from H128 into the periplasm.

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