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Identification of a genetic locus of *Haemophilus influenzae* type b necessary for the binding and utilization of heme bound to human hemopexin

(heme acquisition/bacterial pathogenesis)

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ABSTRACT **The mechanism(s) used by *Haemophilus influenzae* to acquire the essential nutrient heme from its human host has not been elucidated. The heme carried by the high-affinity serum protein hemopexin is one potential source of this micronutrient *in vivo*. A colony-blot assay revealed that heme–human hemopexin-binding activity was shared among most capsular serotype b strains of *H. influenzae* but was uncommon among other strains. We have identified a recombinant clone binding heme–human hemopexin from a *H. influenzae* type b (Hib) genomic library expressed in *Escherichia coli*. Both the Hib strain and the heme–hemopexin-binding clone expressed a polypeptide of ≈100 kDa that bound radiolabeled heme–hemopexin. Oligonucleotide linker insertion mutagenesis of the plasmid DNA from this recombinant clone was used to confirm that expression of the 100-kDa protein correlated with the heme–hemopexin-binding activity. Exchange of one of these mutant alleles into the Hib chromosome eliminated expression of both the 100-kDa protein and the heme–hemopexin-binding activity. Furthermore, this Hib mutant was unable to utilize heme–human hemopexin as a heme source.**

Haemophilus influenzae type b (Hib) strains are responsible for the majority of invasive infections caused by *H. influenzae* (1, 2). Nontypable *H. influenzae* (NTHI) strains are second in importance only to *Streptococcus pneumoniae* as etiologic agents of otitis media in the young (3) and community-acquired bacterial pneumonia in the elderly (4, 5). Although a few surface antigens of *H. influenzae* have been identified as virulence factors (6–8), little attention has been paid to how this parasite satisfies its unusual nutritional requirements in the human host. *H. influenzae* requires exogenously supplied heme and NAD (i.e., the classical X and V factors, respectively) for aerobic growth (9), and it is not known how this pathogen acquires these micronutrients *in vivo*.

Protoporphyrin–iron complexes (i.e., heme) function as the prosthetic groups in proteins required for electron transport and detoxification of oxygen derivatives. (Heme is used in this paper as a generic term describing all iron–protoporphyrin complexes.) Many bacterial pathogens including *H. influenzae* have evolved highly efficient mechanisms for scavenging iron (10–17), and nearly all of these organisms are capable of *de novo* synthesis of protoporphyrin. In contrast, *H. influenzae* is one of the few facultatively anaerobic bacteria that lack the ability to convert δ-aminolevulinic acid to protoporphyrin (18). Therefore, this organism must have developed a mechanism(s) for binding and transport of protoporphyrin (i.e., heme) into the cell. Because heme circulates in the human host in a protein-bound form(s) (19), this mechanism(s) likely involves interaction of these heme–protein complexes with the bacterial cell.

The abundant serum proteins albumin and hemopexin bind heme avidly [K_d values of $\approx 10^{-8}$ and $\approx 10^{-13}$, respectively (20, 21)], and hemoglobin forms a tight complex with haptoglobin (22). It has been shown that *H. influenzae* can utilize all of these heme–protein complexes *in vitro* (23). However, heme bound to albumin or hemopexin cannot be utilized as an iron source by *Neisseria gonorrhoeae* (10), and hemoglobin complexed with haptoglobin is not available as a source of iron to pathogenic *Escherichia coli* (11).

The ability to compete effectively for heme with the heme carrier proteins encountered *in vivo* is, presumably, an important determinant of the virulence of *H. influenzae*. This principle has been demonstrated with respect to the well-characterized iron uptake systems of other bacterial pathogens (24, 25). In contrast, the information available concerning heme acquisition mechanisms in *H. influenzae* is limited (23, 26) although a heme-binding protein of *H. influenzae* that may play a role in heme uptake by this organism has been identified (27). As this protein does not seem to be exposed at the surface of the bacterium (M.S.H., unpublished data), it is unlikely to interact directly with one of the heme carrier proteins.

We used radiolabeled human hemoglobin and heme:human hemopexin as probes of a Hib genomic library in *E. coli* to identify recombinants encoding relevant binding activities. This genetic approach, along with the use of a SDS/PAGE-based blot assay, allowed identification of a heme–human hemopexin-binding protein in Hib. This heme–hemopexin-binding protein was expressed by almost all Hib strains examined but was rare among NTHI strains. A Hib mutant unable to express this protein could not utilize heme–human hemopexin as a source of heme, indicating the essential role of this bacterial protein in acquisition of heme from heme–human hemopexin.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions. The 30 Hib strains and the 20 NTHI strains used in this study have been described (28, 29). Additional *Haemophilus* species used were obtained from F. Sottnek (Centers for Disease Control, Atlanta, GA). *H. influenzae* was grown in brain/heart infusion (BHI) broth supplemented with Levinthal base as a source of heme and NAD (sBHI; ref. 30) with vigorous

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Abbreviations: Hib, *Haemophilus influenzae* type b; NTHI, nontypable *H. influenzae*; BHI, brain/heart infusion; sBHI, supplemented BHI broth; kb, kilobase pairs.

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aeration at 37°C. *H. influenzae* was also grown aerobically on sBHI-agar plates in a candle extinction jar or on BHI-agar containing NAD at 5 μg/ml in an anaerobic chamber (Gaspack, BBL). *E. coli* strains RR1 and HB101 (28) were grown in LB broth or on LB agar, with tetracycline at 15 μg/ml as necessary for plasmid maintenance.

Genetic Techniques. A Hib genomic DNA library was constructed in *E. coli* RR1 as described (27). Plasmids were obtained by a modification of the alkaline lysis method and analyzed by standard restriction enzyme digestion techniques (31). Deletions in the recombinant plasmid pHX1 were obtained by religating *Pst* I partial digestion products and transforming into *E. coli* HB101. Eight base-pair *Not* I linker (GCGGCCGC; New England Biolabs) random insertions into pHX1-6 were generated by a method modified from Heffron *et al.* (32, 38). The method of Herriott *et al.* (33) was used to transform *Bam*HI-linearized pHX1-6::*Not* I linker derivatives into Hib DL42.

Colony-Blot-Binding Assay for Heme Proteins. Human hemoglobin was purchased from Sigma. Human and rat apohemopexin were purified as previously described and then saturated (90%) with heme (34). These proteins were labeled with ^{125}I using the Enzymobead system (Bio-Rad) to specific activities of ≈100 μCi/μg of protein (1 Ci = 37 GBq). Filter preparation, blotting conditions, and buffers were as previously described (35) except that 0.5% (wt/vol) bovine serum albumin was used as the blocking agent, and the radiolabeled heme-carrier proteins were substituted for antibody. *H. influenzae* strains were grown aerobically and anaerobically on sBHI-agar and anaerobically on BHI-NAD-agar for use in the colony-blot system.

SDS/PAGE and Blotting. Proteins in whole cell lysates (29) or cell envelope preparations (27) were fractionated by SDS/PAGE and electrophoretically transferred to nitrocellulose sheets by standard methods (36). Samples in SDS were heated at 37°C for 30 min in the absence of reducing agents prior to PAGE. Nonspecific protein binding sites on the filters were blocked by incubation in phosphate-buffered saline, pH 7.3/0.1% (wt/vol) bovine serum albumin/0.05% (vol/vol) Tween 20. For immunoblotting, filters were probed with rat anti-Hib DL42 serum (27, 29). For blotting with radiolabeled heme–hemopexin, filters were incubated with 2–4 μCi of ^{125}I-labeled heme–hemopexin (^{125}I-heme–hemopexin) overnight at 4°C, washed, dried, and exposed to x-ray film for autoradiography.

Cell Surface Binding Assay. The binding of ^{125}I-heme–human hemopexin to the surface of intact bacterial cells was assessed in an assay adapted from the antibody accessibility assay of Kimura and Hansen (37). Briefly, bacteria grown aerobically on sBHI-agar were incubated with 0.5 μCi of ^{125}I-heme–hemopexin and washed and then the radioactivity of ^{125}I-heme–hemopexin bound to the cells was measured in a γ counter.

Heme Utilization Assay. Cells from an exponentially growing culture of *H. influenzae* in sBHI were sedimented at 12,000 × *g* for 1 min, washed, and then suspended in BHI-NAD. Bacteria were added to molten (56°C) BHI-NAD-agar (0.4% wt/vol) to a final concentration of 10^7 colony-forming units/ml and a 3-ml volume of this suspension was overlaid on a BHI-NAD plate. Filter paper (Whatman 3 MM) discs were placed on the agar and spotted individually with 1–2 μl of each compound being tested as a source of heme. The agar plates were incubated aerobically in a candle jar for 18 hr at 37°C.

RESULTS

Characterization of the Hemopexin-Binding Activity. It has been shown that *H. influenzae* can utilize hemoglobin and heme complexed to human hemopexin as sole sources of heme for aerobic growth *in vitro* (23). We sought to determine whether radiolabeled derivatives of these proteins would bind to *H. influenzae* with sufficient avidity to be useful as probes for cell receptors for heme carrier proteins in a solid-phase assay. In the colony-blot assay, only radioiodinated heme–human hemopexin showed substantial levels of binding to *H. influenzae* cells.

Initial studies with Hib strain DL42 showed that bacteria grown in the absence of heme (anaerobically on BHI-NAD-agar) bound more ^{125}I-heme–human hemopexin than those grown aerobically or anaerobically on sBHI. Fifty *H. influenzae* strains were grown anaerobically in the absence of heme and assayed for their ability to bind ^{125}I-heme–human hemopexin in the colony-blot assay. The majority (25/30) of Hib strains evaluated in this study bound ^{125}I-heme–human hemopexin, while weak ^{125}I-heme–human hemopexin binding was seen only with a few (3/20) NTHI strains (Fig. 1, positions G5, H5, and I4). Heme–human hemopexin-binding activity was not detected among 10 *H. influenzae* strains expressing capsular serotypes a, c, d, e, or f (i.e., two strains of each serotype) or among other species within the genus *Haemophilus* (*H. parainfluenzae, H. avium, H. haemoglobinophilus, H. parahaemolyticus, H. ducreyi, H. aphrophilus, H. paraphrophilus*). *H. influenzae* biotype *aegyptius* expressed weak heme:human hemopexin-binding activity.

Additional experiments showed that both heme-loaded human hemopexin and human apohemopexin were bound apparently to the same extent by these Hib strains in this colony-blot assay (data not shown). However, none of these Hib strains that bound human hemopexin detectably bound radioiodinated rat apohemopexin or heme-loaded rat hemopexin (data not shown).

Identification of a Heme–Human Hemopexin-Binding Protein. Hib DL42 cells were solubilized in 2% SDS at 37°C, and the solubilized components were resolved by SDS/PAGE and electrophoretically transferred to a nitrocellulose filter. Probing the filter with ^{125}I-heme–human hemopexin revealed binding activity in a single band of ≈100 kDa (Fig. 2*A*, lane 1). This band was completely absent in boiled samples (data not shown). One of the Hib strains (Eagan) lacking ^{125}I-heme–human hemopexin-binding activity in the colony blot assay (Fig. 1, position D1) expressed no 100-kDa band as assayed by SDS/PAGE/nitrocellulose blotting and did not express any other heme–human hemopexin-binding proteins detectable in this assay (data not shown).

Molecular Cloning of a Hib Locus Encoding Heme–Human Hemopexin-Binding Activity. DL42, a well-characterized Hib strain that was positive for heme–human hemopexin binding in the colony blot assay (Fig. 1, position B6), was used as the source of DNA for construction of a Hib genomic library in

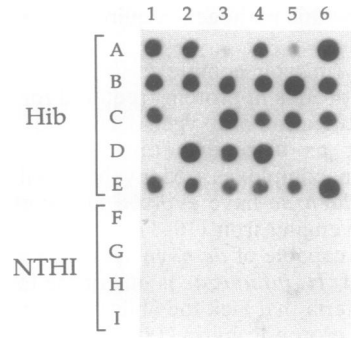

FIG. 1. Colony-blot assay of ^{125}I-heme–human hemopexin binding to *H. influenzae* strains. Colony paste of each anaerobically grown strain was transferred from BHI-NAD agar plates to filter paper with a wooden applicator and blotted with radiolabeled heme–human hemopexin. The 30 Hib strains are arranged in rows A–E; the 20 NTHI strains are in rows F–I (columns 1–5 only).

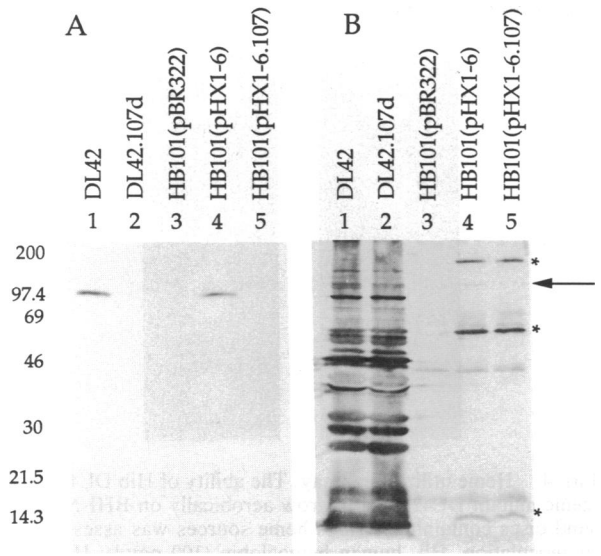

FIG. 2. Analysis of heme–human hemopexin-binding activity and Hib antigens by SDS/PAGE. After resolution by SDS/PAGE, proteins were transferred to nitrocellulose and probed with ^{125}I-heme–human hemopexin (*A*) or rat anti-Hib DL42 serum (*B*) using whole cell lysates (≈5 × 10^8 cells per lane) and cell envelopes (≈80 μg of protein per lane), respectively. Molecular masses (kDa) of protein standards (Rainbow Markers, Amersham) are indicated on the left. The 100-kDa hemopexin-binding protein is indicated by the arrow. Asterisks indicate additional Hib antigens encoded by pHX1-6.

E. coli RR1 (27). Screening of more than 12,000 clones with the colony-blot assay resulted in the identification of a single recombinant that bound ^{125}I-heme–human hemopexin. Restriction enzyme analysis of the recombinant plasmid (pHX1) in this clone revealed that the Hib DNA insert was ≈20 kilobase pairs (kb) in size (Fig. 3). A partial restriction site map of pHX1 was constructed to aid in localization of the gene(s) involved in heme:hemopexin-binding activity. Deletion derivatives of pHX1 were obtained by partial digestion with *Pst* I. The smallest plasmid (pHX1-6) encoding heme:hemopexin-binding activity equivalent to that expressed by pHX1 had an insert of ≈13.8 kb (Fig. 3).

Analysis of whole cell lysates of *E. coli* HB101(pHX1-6) in the SDS/PAGE/nitrocellulose blotting assay revealed the presence of a ^{125}I-heme–human hemopexin-binding protein similar, if not identical, in size to that seen in Hib DL42 (Fig. 2*A*, lanes 1 and 4). As in DL42, the 100-kDa band in HB101(pHX1-6) was absent when the whole cell lysate was treated at 100°C prior to SDS/PAGE. This 100-kDa protein was also detected in an immunoblot assay of *E. coli* HB101(pHX1-6) using anti-Hib DL42 serum (Fig. 2*B*, lane 4). Several additional antigens expressed by pHX1-6 (Fig. 2*B*, lane 4) that are not seen in *E. coli* HB101(pBR322) (Fig. 2*B*, lane 3) were also detected by immunoblotting.

Isolation of Mutants Deficient in Heme–Human Hemopexin-Binding. Plasmid pHX1-6 was randomly linearized by treatment with DNase I/Mn^{2+} (32), ligated to linkers containing a restriction site for *Not* I, and transformed into competent HB101 cells. Of 300 transformants screened in the colony-blot assay, 14 showed decreased binding or a complete lack of ^{125}I-heme–human hemopexin-binding activity. Plasmid DNA was prepared from these mutants and from several heme–hemopexin-binding transformants and digested with *Not* I to confirm insertion of the linker. The approximate positions of 11 linker insertions eliminating or decreasing heme–hemopexin-binding (open arrows) and of 4 insertions having slight or no effect on heme–hemopexin-binding (solid arrows) are shown in Fig. 3. The proteins expressed by these mutants were analyzed by immunoblotting. Those mutants lacking heme–hemopexin-binding activity in the colony blot assay also failed to express a detectable amount of the 100-kDa antigen. In addition, some mutants (Fig. 3, nos. 14 and 15) were detected that exhibited decreased heme–hemopexin-binding and decreased levels of the 100-kDa protein (data not shown); these mutants had no detectable expression of the 60-kDa antigen also encoded on the Hib DNA insert in pHX1-6 (Fig. 2*B*, lane 4).

Deletion subcloning and *Sau*3A digestion techniques were used to localize the regions of the pHX1-6 insert encoding the

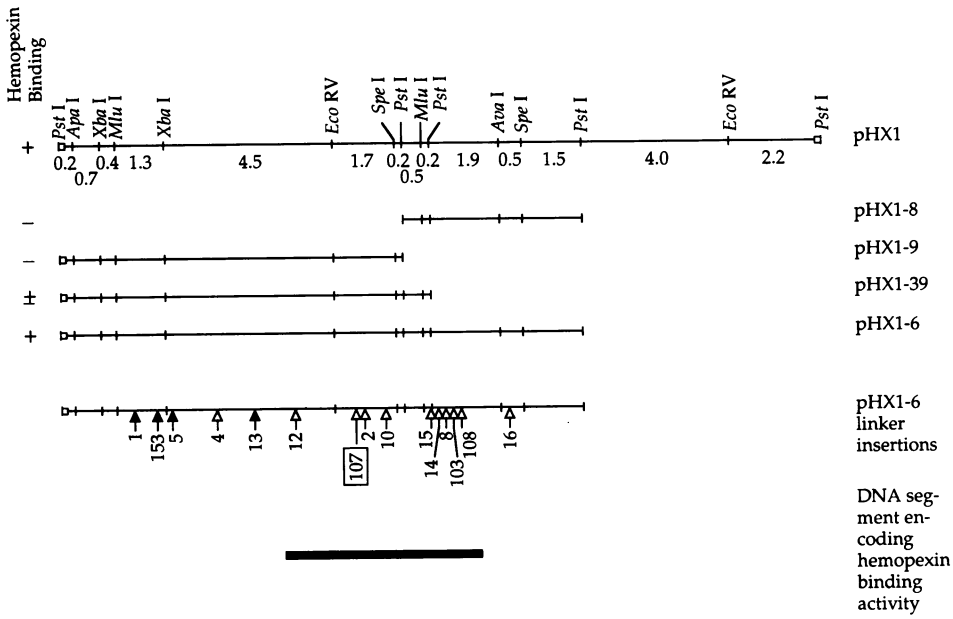

FIG. 3. Partial map of restriction sites and deletion and insertion derivatives of the Hib DNA insert in pHX1. Only the Hib DNA insert portion of pHX-1 is shown; sizes of restriction fragments are indicated in kb. The small open boxes between the ends of the insert and the *Pst* I site of the vector (pBR322) indicate the presence of 15–20 G·C base pairs. The open arrows indicate *Not* I linker insertions into pHX1-6 decreasing or eliminating heme–hemopexin-binding activity; solid arrows indicate positions of linker insertions having slight or no effect on heme:hemopexin binding. The dark bar at the bottom of the figure spans the 5.1-kb DNA segment encoding hemopexin-binding activity.

100-kDa heme:hemopexin-binding protein and the 60-kDa protein described above. Partial *Sau*3A digestion of the 13.8-kb *Pst* I insert in pHX1-6 and subsequent subcloning of the digest into an *E. coli* plasmid vector permitted localization of the DNA encoding the 100-kDa protein to a 5.1-kb fragment extending from ≈1.3 kb 5′ from the *Eco*RV site in pHX1-6 to ≈0.5 kb 5′ from the *Ava* I site in the pHX1-6 insert. It should be noted that almost all of the linker insertions in the pHX1-6 insert that eliminated expression of heme–hemopexin-binding ability mapped in this same 5.1-kb DNA segment (Fig. 3). The 60-kDa protein was expressed by both the recombinant clone containing this 5.1-kb fragment and the pHX1-8 subclone (Fig. 3); these findings indicate that the DNA segment defined by the overlap of the pHX1-8 insert with this 5.1-kb fragment apparently encodes this 60-kDa protein.

The *E. coli* recombinant containing the mutated plasmid pHX1-6.107 (Fig. 3) was selected as an example of a mutant lacking heme–hemopexin-binding activity by SDS/PAGE/nitrocellulose blotting (Fig. 2*A*, lane 5) and lacking only the 100-kDa insert-encoded antigen (Fig. 2*B*, lane 5). This mutated plasmid was linearized by cutting at the single *Bam*HI site in the vector and used to transform Hib DL42 to introduce, by homologous recombination, the mutation in the cloned heme–hemopexin-binding locus into the Hib chromosome. Among the transformants that were obtained on sBHI agar, several failed to bind ^{125}I-heme–human hemopexin in the colony blot assay. Analysis of one of these, Hib DL42.107d, is shown in Fig. 2. SDS/PAGE/nitrocellulose blot analysis confirmed that Hib DL42.107d failed to express the 100-kDa heme–hemopexin-binding species (Fig. 2*A*, lane 2). The presence of other Hib proteins with a similar molecular mass in the whole cell lysate precluded demonstration of the absence of the 100-kDa antigen in Hib DL42.107d by immunoblotting with anti-DL42 serum (Fig. 2*B*, lane 2).

Surface Localization of the Heme–Hemopexin-Binding Protein. The ability of viable and presumably intact Hib cells harvested from agar plates to bind ^{125}I-heme–human hemopexin and remove it from suspension is one measure of the surface accessibility of the 100-kDa protein. Both Hib DL42 and *E. coli* HB101(pHX1-6) bound ^{125}I-heme–human hemopexin in liquid phase (19,650 and 10,950 cpm, respectively). The *E. coli* linker mutant HB101(pHX1-6.107) and the Hib mutant DL42.107d bound ^{125}I-heme–hemopexin (373 cpm and 383 cpm, respectively) at levels similar to that obtained with the *E. coli* strain containing only the vector [HB101(pBR322), 439 cpm].

Heme–Human Hemopexin Utilization by Hib. We sought to determine whether the 100-kDa protein played a role in the uptake of heme by Hib. The ability of the wild-type Hib parent strain DL42 and the Hib mutant DL42.107d to utilize hemoglobin, heme–human hemopexin, and free heme (hemin) as sources of heme was assessed by the presence of bacterial growth on heme-free medium around discs containing these compounds (Fig. 4). Both Hib strains failed to grow in the absence of heme (P, phosphate-buffered saline), and showed equivalent growth utilizing human hemoglobin (Hb, 100 pmol) or equine hemin (H, 1 nmol). However, heme–hemopexin (Hx, 100 pmol) served as a source of heme only for the wild-type strain and not for the mutant lacking the 100-kDa protein.

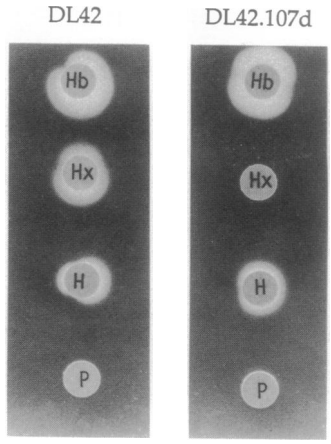

FIG. 4. Heme utilization assay. The ability of Hib DL42 and the isogenic mutant DL42.107d to grow aerobically on BHI-NAD agar around discs containing various heme sources was assessed 18 hr after inoculation. Hb, human hemoglobin (100 pmol); Hx, heme–human hemopexin (100 pmol); H, hemin (equine, 1000 pmol); P, phosphate-buffered saline (negative control).

DISCUSSION

The requirement of some *Haemophilus* species (39–41) for the porphyrin moiety provided by heme is shared only by anaerobic *Bacteroides* species (42–44) and certain parasitic protozoans such as *Leishmania* (45). The Hib heme–human hemopexin-binding protein described in this study is, so far as we know, the first example of a macromolecule that has been shown to be required for heme utilization by a bacterial pathogen. The highly efficient heme–human hemopexin scavenging system of Hib is even more remarkable in this context as cultured primary hepatocytes, the cells responsible for most *in vivo* heme uptake and catabolism, are apparently inefficient at removing heme from hemopexin (46).

In nature, *H. influenzae* is found only in humans. This complicates determination of the true *in vivo* source(s) of heme for this pathogen. Possibly the propensity of *H. influenzae* to disseminate to various tissues within its human host is determined, in part, by its ability to acquire heme from the different heme carrier proteins. The segregation of heme–hemopexin-binding activity mainly to Hib strains, which are invasive, and not to NTHI strains, which are primarily mucosal surface pathogens, is suggestive in this regard. In this context, it is noteworthy that there is a marked increase in levels of hemopexin in serum that takes place between 2 and 6 months after birth (47). This change in hemopexin abundance in the serum occurs during the same time period in which there is a dramatic increase in the incidence of invasive Hib disease, an occurrence usually attributed to the waning of protective levels of maternal antibodies (48).

The Hib DL42 genetic locus expressing heme–human hemopexin-binding activity also encodes several other proteins detectable by Western blot analysis in addition to the 100-kDa protein (Fig. 2*B*, lane 4). The possible involvement of at least one of these proteins in either expression of the 100-kDa protein or in the binding of heme–hemopexin to the bacterial cell surface is suggested by results obtained from the linker mutagenesis experiments. Linker insertions (Fig. 3, nos. 14 and 15) that eliminated expression of the 60-kDa immunoreactive protein encoded by the Hib DNA insert in pHX1-6 exerted a negative effect on expression of the 100-kDa protein and on heme–hemopexin-binding ability (data not shown). These results, and the diminished heme–hemopexin-binding activity of the pHX1-39 subclone (Fig. 3) that also cannot express the 60-kDa protein (data not shown), imply that expression of the 100-kDa protein is necessary but not sufficient for maximal heme–hemopexin binding.

The fact that Hib strains bind human hemopexin but not rat hemopexin indicates the existence of some degree of species specificity in the binding of hemopexin by the Hib 100-kDa protein. A practical consequence of this lack of reactivity of the 100-kDa protein with rat hemopexin is that it precludes the use of the infant rat model for invasive Hib disease (49) to evaluate the possible role of the 100-kDa protein in the

expression of virulence by this pathogen. In fact, a mutant of Hib strain DL42, constructed by transposon-mediated mutagenesis, that lacks expression of both the 100-kDa and the 60-kDa proteins encoded by pHX1-6 was found to be as virulent as the wild-type parent strain in this infant rat model (data not shown). This latter result indicates clearly that Hib DL42, which cannot bind heme–rat hemopexin to its cell surface, possesses alternative mechanisms for obtaining heme when growing *in vivo* in infant rats.

The ability of the Hib mutant lacking the 100-kDa protein to utilize hemoglobin as a source of heme *in vitro* (Fig. 4) indicates that in Hib DL42 there exists a separate mechanism(s) for acquiring this iron–protoporphyrin complex from hemoglobin. Therefore, *H. influenzae* must possess at least two different systems for obtaining heme from human hemoglobin and from heme–human hemopexin. In addition, the recombinant *E. coli* strains that bound heme–human hemopexin [i.e., RR1(pHX1)] did not bind hemoglobin, a finding implying that the Hib locus that encodes the 100-kDa heme–human hemopexin-binding protein does not encode any proteins directly involved in the binding of hemoglobin.

It is important to note that NTHI strains, which utilize heme–human hemopexin as a source of heme *in vitro* (23), did not bind heme–human hemopexin in the colony-blot system (Fig. 1). We have confirmed that NTHI strains and Hib strain Eagan [which did not bind heme–human hemopexin in the colony-blot system (Fig. 1, position D1)], can grow *in vitro* using heme–human hemopexin as the sole source of heme (data not shown). These results indicate that these particular *H. influenzae* strains possess a mechanism for obtaining heme from heme–human hemopexin that does not involve the 100-kDa protein characterized in the present study. This apparent multiplicity of heme acquisition systems in *H. influenzae* is consistent with the importance of this micronutrient to the ability of this pathogen to parasitize successfully its human host.

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