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The heme-binding protein (HbpA) of *Haemophilus influenzae* as a virulence determinant

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Abstract

Haemophilus influenzae has an absolute growth requirement for heme and the heme-binding lipoprotein (HbpA) and has been implicated in the utilization of this essential nutrient. We constructed an insertional mutation of *hbpA* in a type b and a nontypeable *H. influenzae* strain. In the type b strain, the *hbpA* mutant was impaired in utilization of heme complexed to either hemopexin or to albumin and in the utilization of low levels of heme but not in the utilization of heme at high levels or of hemoglobin or hemoglobin–haptoglobin complexes. In contrast, the *hbpA* mutant derivative of the nontypeable strain was impaired in utilization of all tested heme sources. We further examined the impact of the *hbpA* mutation in animal models of *H. influenzae* disease. The *hbpA* mutant of the nontypeable strain was indistinguishable from the wild-type strain in the chinchilla model of otitis media. The *hbpA* mutant derivative of the type b strain caused bacteremia as well as the wild-type strain in 5-day old infant rats. However, in 30-day old rats the *hbpA* caused significantly lower rates of bacteremia than the wild-type strain indicating a role for *hbpA* and heme acquisition in virulence in this model of *H. influenzae* disease. In conclusion, HbpA is important for heme utilization by multiple *H. influenzae* strains and is a virulence determinant in a model of *H. influenzae* invasive disease.

Keywords

Haemophilus influenzae; Heme; Virulence

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Introduction

Haemophilus influenzae is a fastidious facultatively anaerobic Gram-negative bacterium that causes a range of human infections including otitis media, meningitis, epiglottitis, and pneumonia (Turk, 1984). While infections caused by encapsulated type b strains have been largely eradicated in the developed world following introduction of a vaccine based on the type b capsule (Kelly et al., 2004), nontypeable *H. influenzae* (NTHi) strains continue to be a significant cause of otitis media and pneumonia. Since *H. influenzae* lacks all enzymes in the biosynthetic pathway for the porphyrin ring, it is unable to synthesize protoporphyrin IX (PPIX), the immediate precursor of heme. It has an absolute growth requirement for an exogenous source of PPIX or heme during aerobic growth (Panek et al., 2002; White et al., 1963). The only known niche for *H. influenzae* is man, and potential heme sources in the human host are limited. Heme is generally intracellular, in the form of hemoglobin or heme-containing enzymes (Griffiths, 1999). Extracellular hemoglobin, derived from lysed erythrocytes, is bound by the serum protein haptoglobin, and the hemoglobin–haptoglobin complex is rapidly cleared by the reticuloendothelial cells of the liver, bone marrow, or spleen (Evans et al., 1999; Ward et al., 1999). Similarly, free heme, principally derived from the degradation of methemoglobin, is bound by the serum proteins hemopexin and albumin and cleared from the circulation (Evans et al., 1999; Peters, 1996; Ward et al., 1999). Hemoglobin and the hemoglobin–haptoglobin, heme–hemopexin, and heme–albumin complexes as well as PPIX in the presence of an iron source can be utilized by *H. influenzae* as heme sources in vitro (Morton et al., 1989; Stull, 1987). *H. influenzae* has evolved a complex multifunctional array of uptake mechanisms to ensure that it is able to utilize available porphyrin in vivo (Morton et al., 2004c).

One protein involved in heme utilization is the heme-binding lipoprotein HbpA (Hanson et al., 1991, 1992; Morton et al., 2005). HbpA was initially identified as a potential constituent of a heme acquisition pathway following transformation of an *H. influenzae* genomic DNA library into *Escherichia coli* and screening for recombinant clones with heme-binding activity (Hanson et al., 1991). Expression of heme-binding activity by *E. coli* correlated with the expression of a protein of approximately 51 kDa, sized on SDS-PAGE gels, that was subsequently purified in a heme–agarose affinity purification protocol, from both recombinant *E. coli* and *H. influenzae*, and shown to be a lipoprotein (Hanson et al., 1991). Additionally HbpA was localized to the periplasmic space and shown to be associated with both the inner membrane and the outer membrane in *H. influenzae* (Hanson et al., 1991, 1992). The authors proposed that HbpA may serve to transport heme into the cytosol of *H. influenzae* subsequent to initial binding steps at the cell surface (Hanson et al., 1991, 1992). Subsequently, HbpA was shown to be involved in the utilization of heme from various sources in a NTHi strain (Morton et al., 2005). The goal of this study was to extend the investigation of the role of HbpA in heme utilization to an *H. influenzae* type b (Hib) strain and to determine if HbpA is important for virulence in animal models of *H. influenzae* disease.

Materials and methods

Bacterial strains and growth conditions

NTHi strain 86-028NP is a minimally passaged clinical isolate from a pediatric patient who underwent tympanostomy and tube insertion for chronic otitis media at Columbus Children's Hospital. Strain 86-028NP has been extensively characterized in chinchilla models of otitis media and nasopharyngeal colonization (Bakaletz et al., 1999; Kennedy et al., 2000; Morton et al., 2004a; Suzuki et al., 1994). Hib strain HI689 is a clinical isolate from a patient with bacteremia and has been extensively characterized with respect to heme utilization and in rat models of bacteremia (Morton et al., 1999, 2004b; Musser et al., 1986; Seale et al., 2006). *H. influenzae* were routinely maintained on chocolate agar with bacitracin (BBL, Becton-

Dickinson, Sparks, MD, USA) at 37°C. When necessary, *H. influenzae* were grown on brain heart infusion (BHI) agar (Difco, Becton-Dickinson) supplemented with 10 μg ml^{-1} heme and 10 μg ml^{-1} β-NAD (supplemented BHI; sBHI) and the appropriate antibiotic(s). Heme-deplete growth was performed in BHI broth supplemented with 10 μg ml^{-1} β-NAD alone (heme-deplete BHI; hdBHI). Spectinomycin was used at 200 μg ml^{-1} in *H. influenzae*.

Heme sources

Human hemoglobin, human haptoglobin, human serum albumin (HSA), and heme (as hemin) were purchased from Sigma. Stock heme solutions were prepared at 1 mg ml^{-1} heme in 4% v/v triethanolamine as previously described (Poje et al., 2003). (Heme is correctly defined as ferrous PPIX while hemin is ferric PPIX. However, for the purposes of this manuscript, heme is used as a general term and does not indicate a particular valence state). Hemoglobin was dissolved in water immediately before use. Hemoglobin–haptoglobin complexes were prepared as previously described (Morton et al., 1999). Heme–albumin complexes were made by mixing 100 μg heme and 20 mg HSA per ml of water as previously described (Stull, 1987).

Rabbit hemopexin was prepared as described previously, and the heme–hemopexin complexes were characterized by the typical features of their absorption spectra, which include the prominent shoulder at 290 nm that appears upon heme binding (Smith et al., 1984; Smith, 1985).

Construction of *hbpA* insertion mutants

Insertional mutations of *hbpA* in strains HI689 and 86-028NP were made using the mutagenic construct plasmid (pDJM346) previously described for mutation of *H. influenzae* nontypeable strain HI1388 (Morton et al., 2005). Briefly, pDJM346 was constructed as follows: A 2776-bp region encompassing *hbpA* was amplified using the PCR and cloned into the TA cloning vector pCR2-1-TOPO to form pDJM1 (Morton et al., 2005). The spectinomycin cassette from pSPECR (Whitby et al., 1998) was excised with *Eco*RV and cloned into the unique *Pml*I (internal to *hbpA*) of pDJM1 to yield pDJM346 (Morton et al., 2005). Competent *H. influenzae* were transformed to spectinomycin resistance with pDJM346, using the static aerobic method as previously described (Morton et al., 2004a), and selected on sBHI agar containing spectinomycin. Correct chromosomal recombinations were confirmed by the molecular size of a PCR product resolved on an agarose gel (data not shown).

Growth studies with *H. influenzae*

Growth studies were performed using the Bioscreen C Microbiology Reader (Oy Growth Curves AB Ltd., Helsinki, Finland) as previously described (Morton et al., 2005, 2006a).

Chinchilla model of otitis media

A total of 16 adult chinchillas (*Chinchilla lanigera*) with no evidence of middle ear infection by either otoscopy or tympanometry at the beginning of the study were used. Animals were rested for 7–10 days upon arrival to acclimate them to the vivarium. After acclimation, chinchillas were challenged with either NTHi strain 86-028NP or the *hbpA* mutant derivative HI2092 both intranasally and transbullarly with approximately 10^8 or 2500 c.f.u., respectively. Intranasal inoculation was performed by passive inhalation of a bacterial suspension delivered to the nares of chinchillas that were lightly anesthetized and were lying in a prone position. Transbullar inocula were delivered by direct injection of bacterial suspensions into the superior bullae. Actual challenge dosages received were confirmed by plate count. Animal procedures have been described in detail elsewhere (Bakaletz et al., 1997, 1999; Gitiban et al., 2005).

Two individual experiments were performed on 2 different time schedules. In the first experiment, 2 groups of 3 animals each were challenged with 86-028NP and HI2092, respectively, and sampling performed on days 1, 4, 7, 10, 14, and 18 post-challenge. In the second experiment, groups of 5 animals were challenged with 86-028NP and HI2092, and sampling was performed on days 1, 2, 3, and 4 post-challenge.

Animals were blindly evaluated by otoscopy and tympanometry at least every 2 days from the time of bacterial inoculation until completion of the experiment. Signs of tympanic membrane inflammation were evaluated by video otoscopy (Video VetScope System, MedRx, Seminole, FL, USA) and were rated on a 0 to 4+ scale. Tympanometry (EarScan, South Daytona, FL, USA) was used to monitor changes in middle ear pressure, tympanic width, and tympanic membrane compliance as previously described (Bakaletz et al., 1999; Kennedy et al., 2000).

Nasopharyngeal lavage was performed on all animals by passive inhalation of 500 μl pyrogen-free, sterile saline, delivered dropwise to one nares, with collection of drainage from the contralateral nares. Epitympanic taps were performed on the same schedule as nasopharyngeal lavages and occurred whenever an effusion was considered to be of sufficient volume to be retrieved (i.e. any ears scored as 2.5 or greater for inflammation). All recovered nasopharyngeal lavage and epitympanic tap fluids were maintained on ice until serially diluted. Dilutions of nasopharyngeal lavage fluids were then cultured on chocolate agar plates containing bacitracin (BBL) to select for *H. influenzae*. Dilutions of epitympanic tap fluids were cultured on chocolate agar plates (BBL). All plates were incubated at 37°C for 48 h to quantify c.f.u. NTHi/ml. Animals were also tabulated as having a ‘colonized’ or ‘cleared’ status based on culture results as previously described (Bakaletz et al., 1999; Kennedy et al., 2000).

Rat model of bacteremia

Specified pathogen free (SPF), timed-pregnant Sprague-Dawley rats (Harlan, Indianapolis, Ind., USA) were received approximately 5 days prior to giving birth. Newborn pups from different mothers were pooled and randomly reassigned to the mothers (n=10 pups per female). Pups were weaned at 21 days.

The rat model for hematogenous meningitis following intraperitoneal infection with *H. influenzae* (Smith et al., 1973) was used to compare the abilities of strains to cause bacteremia in both 5-day old and 30-day old rats.

Inocula were prepared from broth cultures incubated as previously described (Seale et al., 2006). Bacterial suspensions were pelleted by centrifugation, washed once with phosphate buffered saline (PBS) containing 0.1% gelatin, and then resuspended in the same buffer. The suspension was adjusted to an A_{605} = 0.50 and then diluted serially in the same solution to provide the standard inoculum (200 c.f.u. in 100 μl for experiments in 5-day old rats and 2000 c.f.u in 100 μl for experiments in 30-day old rats) for intraperitoneal injection into the rats. The actual infective dosage was confirmed in each experiment by plating on chocolate agar containing bacitracin. Colony counts were made after the plates were incubated at 37°C for 24 h. At specified time points, blood specimens (50 μl) were obtained from anesthetized animals (gaseous Halothane, Halocarbon Laboratories, River Edge, N.J., USA) by cardiac puncture (Smith et al., 1973). The method used for quantifying bacteremia caused by the infection of infant rats with *H. influenzae* was based on the track-dilution procedure of Jett et al. (1997) and Morton et al. (2004b). Serial dilutions (0 to 10^{-5}) of whole blood specimens freshly drawn in heparinized syringes were made with PBS containing 0.1% gelatin. Aliquots of 10 μl from each dilution were plated in triplicate on sBHI agar in square polystyrene plates (100×15 mm) with grids (Fisher Scientific). Colonies were counted after incubation at 37°C for 24 h. Clearance or failure to establish bacteremia was confirmed by plating five 10-μl aliquots of

heparinized blood on at least 2 subsequent days. The sensitivity limit for the detection of bacteremia was 20 c.f.u./ml blood.

Statistics

Statistical comparisons of growth between strains under the same growth conditions in vitro were made using the Kruskal-Wallis test. Some analyses were made over the active period of growth only. Analyses were performed using Analyse-It for Microsoft Excel v1.71 (Analyze-It Software Inc., Leeds, England). A p-value <0.05 was taken as statistically significant.

For studies in the infant rat model of invasive disease, bacteremic titers are expressed as mean values ± SD typically from groups of 10 animals. Bacterial titers in blood were compared between groups of rats challenged with either the wild-type *H. influenzae* strain HI689 or the corresponding *hbpA* mutant strain HI1886, using the Kruskal-Wallis test (Analyse-It).

Percentages of bacteremic pups and weights of bacteremic pups infected with wild-type or mutant strains were compared by the Fisher Exact Test. Analyses were performed with SigmaStat software (SPSS Inc., Chicago, Ill., USA). A p-value <0.05 was taken as statistically significant. Bacterial loads in middle ear effusions and in nasopharyngeal lavages from groups of chinchillas were compared using a Wilcoxon Rank Sum test. A p-value <0.05 was taken as statistically significant.

Results and discussion

Growth characteristics of *H. influenzae hbpA* mutants

We have previously shown that the heme-binding lipoprotein HbpA is involved in utilization of heme from multiple heme sources in the nontypeable strain HI1388 (Morton et al., 2005). However, we were unable to assess the potential role of HbpA in virulence in animal models in this background. In preliminary experiments, strain HI1388 did not cause bacteremia in the infant rat model of invasive disease and in the chinchilla model of otitis media infection progressed rapidly to labyrinthitis, a criterion for termination of the animal protocol (data not shown). As a result of these observations, 2 strains that have been well characterized in these animal models were selected for further studies. Mutations in the *hbpA* genes of the nontypeable strain 86-028NP and the type b strain HI689 were successfully constructed and confirmed by PCR (data not shown). Growth studies were performed to assess the impact of the *hbpA* mutation on heme source utilization in these strains. The *hpbA* mutant derivative of 86-028NP (designated HI2092) exhibited a reduced ability to utilize heme, heme–albumin complexes, hemoglobin, and hemoglobin–haptoglobin complexes at all tested concentrations compared to the wild-type strain (Fig. 1). Fig. 1A shows growth of strain 86-028NP and its *hbpA* mutant derivative in varying concentrations of free heme and demonstrates that at all concentrations the mutant grew significantly less well than the wild-type strain (p<0.0001). Utilization of heme–human serum albumin complexes (200 ng ml^{-1} heme equivalent), hemoglobin 10 μg ml^{-1} and hemoglobin–haptoglobin complexes (10 μg ml^{-1} hemoglobin equivalent) is shown in Fig. 1B, in all cases utilization of the heme source was significantly reduced in the mutant strain HI2092 compared to the wild-type strain 86-028NP (p<0.0001 for all tested heme sources at all tested concentrations). These data confirm the findings reported for the *hbpA* mutant of the nontypeable strain HI1388 which similarly showed a reduced ability to utilize the aforementioned heme sources (Morton et al., 2005). In the case of the HI1388 *hbpA* mutant derivative, there was also a significantly reduced ability to utilize heme–hemopexin (Morton et al., 2005). In the case of 86-028NP, the wild-type strain was unable to use heme–hemopexin as a heme source (data not shown). It is not clear why strain 86-028NP is unable to utilize heme–hemopexin since utilization of heme–hemopexin complexes is mediated by the *hxuCBA* gene cluster (Cope et al., 1995; Morton et al., 2007a), and the genome of strain

86-028NP contains an intact *hxuCBA* gene cluster (Harrison et al., 2005). It should also be noted that strain 86-028NP retains the ability to utilize the heme–human serum albumin complex as a sole heme source. In several *H. influenzae* strains HxuC mediates utilization of heme–albumin complexes as a heme source whereas neither HxuA nor HxuB are required for utilization of this heme source (Morton et al., 2007a). Thus, the failure to use heme–hemopexin complexes by 86-028NP probably does not result from an inactive HxuC protein.

In contrast to the data reported above for strain 86-028NP and that previously reported for strain HI1388 (Morton et al., 2005), mutation of *hbpA* in the type b strain HI689 impacted acquisition of heme from a more limited range of heme sources. When grown in heme–albumin at concentrations of 50 or 25 ng ml^{-1} heme equivalent, the mutant grew less well than the wild-type strain (Fig. 2A; $p<0.0001$) as evidenced by both a delayed onset of growth and a reduced growth rate. Although the mutant strain appeared to have a delayed onset of growth compared to the wild-type strain in heme–albumin at 100 ng ml^{-1} heme equivalent (Fig. 2A), there was no significant difference when the mutant strain and the wild type were compared over the entire growth period ($p=0.4156$). However, when compared over the period of active growth (i.e. between hours 6 and 18) there is a significant difference in growth between the mutant and wild-type strains when grown in heme–albumin at 100 ng ml^{-1} heme equivalent (Fig. 2A; $p=0.0036$). Additionally, when heme was supplied as heme–hemopexin, the *hbpA* mutant strain HI1886 grew significantly less well than the wild-type strain HI689 at all tested concentrations (Fig. 2B; $p<0.0001$ at all concentrations). An impact of the *hbpA* mutation on utilization of heme by strain HI689 became apparent only at low concentrations. When grown in either 2 or 1 μg ml^{-1} heme, there was no significant difference between growth of the mutant and wild-type strain ($p=0.6737$ and $p=0.0966$, respectively). However, at either 0.5 or 0.25 μg ml^{-1}, heme growth of the mutant was significantly impaired compared to the wild type ($p<0.0001$). Growth of the *hbpA* mutant in either hemoglobin or hemoglobin–haptoglobin was indistinguishable from that of the wild-type strain at all tested concentrations [see Fig. 2B for growth in hemoglobin–haptoglobin 2 μg ml^{-1} hemoglobin equivalent ($p=0.3213$); for additional concentrations $p=0.3396$ in 10 μg ml^{-1} hemoglobin, $p=0.7792$ in 5 μg ml^{-1} hemoglobin, $p=0.9414$ in hemoglobin–haptoglobin 10 μg ml^{-1} hemoglobin equivalent, $p=0.7462$ in hemoglobin–haptoglobin 5 μg ml^{-1} hemoglobin equivalent, and $p=0.3997$ in hemoglobin–haptoglobin 0.5 μg ml^{-1} hemoglobin equivalent]. Complementation of mutant strains with a plasmid-borne copy of the wild-type *hbpA* gene corrected the phenotypic changes seen in *H. influenzae hbpA* mutants (data not shown) (Morton et al., 2005).

These data indicate that strains of *H. influenzae* possess other periplasmic heme transport systems in addition to HbpA. Although these additional periplasmic heme transport systems have not been defined to date, there are at least 2 promising candidates that warrant further study. One such candidate is the *hip* operon which encodes a putative ABC transporter and has been shown in preliminary studies to be involved in heme utilization (VanWagoner et al., 2007). The *hip* locus is intact only in type b strains and is present only as a remnant in most strains (VanWagoner et al., 2007). This difference in distribution of the *hip* locus may in part account for the clear differences in the impact of the *hbpA* mutation between the type b strain and the nontypeable strains reported herein and in our previous report (Morton et al., 2005). An additional locus potentially involved in periplasmic heme transport is the *sap* operon. The *sap* operon is involved in resistance to antimicrobial peptides (Mason et al., 2006), and preliminary studies also indicate a potential role in heme utilization (Mason et al., 2008). Two additional putative periplasmic proteins are homologous to both HbpA and SapA and may be involved in heme acquisition; these 2 putative proteins are encoded by the ORFs designated HI0213 and HI1124 in the *H. influenzae* strain Rd KW20 genomic sequence (Fleischmann et al., 1995). In a microarray study of the response of Rd KW20 to iron and heme levels in the growth media, the ORF HI0213 was maximally transcribed under conditions of iron/heme restriction, supporting a potential role in heme acquisition (Whitby et al., 2006). However, in

a study of 2 additional *H. influenzae* strains transcription of the HI0213 homolog in those strains was unaffected by iron/heme levels (Whitby et al., 2009). There is no empirical data reported to date to support a role for either HI0213 or HI1124 in heme utilization, and any association remains speculative. Further studies are required to elucidate the full range of genes involved in transport of heme across the periplasm in *H. influenzae*.

Role of *hbpA* in *H. influenzae* virulence

We compared the *hbpA* mutant derivatives of strains 86-028NP and HI689 for their abilities to establish and maintain infection in animal models of *H. influenzae* disease.

The nontypeable *H. influenzae* strain 86-028NP was compared with the *hbpA* mutant derivative HI2092 in the chinchilla model of otitis media and nasopharyngeal colonization. Two separate experiments were performed over 2 different time courses. In the first experiment, 2 groups of 3 animals each were challenged with the wild-type and mutant strains, respectively, and assessed as described in the 'Materials and methods' on days 1, 4, 7, 10, 14, and 18 post-challenge. Using video otoscopy and tympanometry, no differences were observed in the progression of otitis media between the 2 groups of animals. In addition, there was no statistical difference in bacterial load in ear effusions recovered from the 2 groups of animals at any time point (data not shown). With respect to nasopharyngeal colonization, there was an apparent trend towards reduced bacterial load in nasopharyngeal lavages from early time points. On day 1 post-challenge, the mean bacterial load (±SD) in nasopharyngeal lavages from the wild-type strain was $1.4\times10^5 \pm 2.3\times10^5$ cfu/ml and that for the mutant was $7.5\times10^3 \pm 1.2\times10^4$ cfu/ml, corresponding numbers on day 2 post-challenge were $5.9\times10^5 \pm 7.3\times10^5$ cfu/ml and $4.8\times10^3 \pm 1.4\times10^3$ cfu/ml, respectively. However, since each group contained only 3 animals, it was not possible to determine if the differences observed at early time points were statistically significant at $p<0.05$. At subsequent time points, there was no difference between bacterial loads in nasopharyngeal lavages between the 2 groups.

In view of these results, a second experiment was performed in which 2 groups of 5 animals each were challenged with the wild-type and mutant strain, respectively, and assessed on days 1, 2, 3, and 4 post-challenge (middle ear effusions could not be recovered on day 1 post-challenge). At no time point was there any statistically significant difference between the 2 groups with respect to bacterial load in either ear effusions or nasophryngeal lavage samples (data not shown). In addition, no differences were observed between the 2 groups by either video otoscopy or tympanometry. These data indicate that *hbpA* is not required for nasopharyngeal colonization or for infection of the middle ear in the chinchilla models of *H. influenzae* colonization and otitis media. The failure to detect a role for HbpA in virulence in the chinchilla model likely reflects the presence of additional periplasmic heme uptake systems that substitute for the role of HbpA. The failure of these putative additional periplasmic heme uptake systems to fully substitute for HbpA in our in vitro studies does not preclude this possibility for a number of reasons. We do not currently have a complete understanding of the heme sources available to *H. influenzae* in either the middle ear or the nasopharynx, and it is possible that there is an (or are) as yet unidentified heme source(s) whose utilization is unimpaired in a mutant lacking HbpA. Thus, a more complete understanding of the heme sources available in both the middle ear and the nasopharynx is necessary before a comprehensive explanation for the lack of an apparent phenotype in these models of infection can be made.

One possible alternative interpretation of these data from the chinchilla experiments is that heme is not required during middle ear infection. However, in a previous study we have shown that a strain 86-028NP mutant lacking the hemoglobin/hemoglobin–haptoglobin binding proteins (Hgps) causes less severe middle ear infection than does the wild-type strain, indicating that heme acquisition is required during otitis media (Morton et al., 2004a). Deletion

of the Hgps completely abrogates utilization of hemoglobin–haptoglobin in vitro (Morton et al., 1999, 2004a), whereas mutation of *hbpA* reduces but does not completely abolish utilization of any source. In addition, it is possible that during otitis media the heme requirement of *H. influenzae* is significantly less than during aerobic growth in vitro, and thus the impact of an *hbpA* mutation would be less deleterious.

The *hbpA* mutant strain of the type b strain (HI1886) was additionally compared to its wild-type progenitor (HI689) for its ability to establish and maintain bacteremia in both 5-day old and 30-day old rats. In 5-day old rats, both strains were equally able to establish infection. All rats infected with either strain HI689 (n=10) or HI1886 (n=10) exhibited bacteremia within 24 hours. Bacteremia persisted in all rat pups for the 7 days of the assessment period and bacteremic titers in all pups were comparable at all time points (data not shown). In addition, there was no difference in weight gain between the cohort infected with strain HI689 and that infected with strain HI1886 (data not shown). Weight gain is a non-specific but quantitative measure of well-being, and the failure to detect a difference in weight gain indicates that there is no difference between the virulence of the 2 strains.

In 30-day old rats the wild-type strain more readily established infection than did the mutant strain (Fig. 3). On day 1, 10 of 10 rats infected with HI689 were bacteremic while only 5 of 10 rats infected with HI1886 had a detectable bacteremia ($p<0.02$). On days 2 and 3 post-infection, 10 of 10 rats infected with strain HI689 remained bacteremic while 3 of 10 rats infected with HI1889 still had a detectable bacteremia ($p<0.0003$). By day 7 post-infection, all rats infected with strain HI1886 had cleared the bacteria while 90% of those infected with HI689 still had a detectable bacteremia ($p<0.0001$). Rosadini et al. (2008) have recently reported that mutation of *hbpA* in the type d derivative strain Rd KW20 resulted in reduced survival of the mutant strain compared to the wild-type strain 24 hours following intraperitoneal inoculation of 6.5-week old mice. Strain Rd KW20 is not a classically virulent *H. influenzae* strain, and this experiment required the use of an infective dose of approximately 4×10^8 bacteria, a dose significantly larger than that required for typically invasive strains of *H. influenzae* (i.e. the 2000-fold c.f.u. dose used in this study for 30-day old rats). The data reported by Rosadini et al. (2008) are consistent with a role for *hbpA* in bacteremia in adult animals.

Similar age-related differences in the impact on bacteremia in the rat model to those reported here for mutation of *hbpA* have been reported by us for other genes associated with heme acquisition, including those encoding the lipoprotein (*e*) P4, the hemoglobin/hemoglobin–haptoglobin-binding proteins and the heme–hemopexin/heme–albumin utilization proteins HxuCBA (Morton et al., 2007a, b; Seale et al., 2006). These observations may reflect changes in the available heme sources in the aging rat. For example, haptoglobin levels in rat plasma decrease immediately following birth, reaching a nadir at 5 days of age and subsequently increasing to reach adult levels by 30 days of age (Seale et al., 2006). Hemopexin levels also change in the developing rat (Moldenhauer et al., 1970). The predominant heme sources available during invasive disease are likely to be hemoglobin, hemoglobin–haptoglobin, and heme–hemopexin. However, other sources may also be available, including catalase and myoglobin–haptoglobin complexes both of which are utilized as heme sources by *H. influenzae* in vitro (Morton et al., 2006b, 2008). It is possible that additional as yet unidentified heme sources are also present. Thus, the full range of heme sources available to *H. influenzae* during bacteremia in the rat model remains to be fully defined.

In conclusion, we have shown that mutation of *hbpA* has a differential phenotypic impact with respect to the utilization of various heme sources in nontypeable and type b *H. influenzae* and that *hbpA* is important during bacteremia in the weanling rat model of *H. influenzae* disease.

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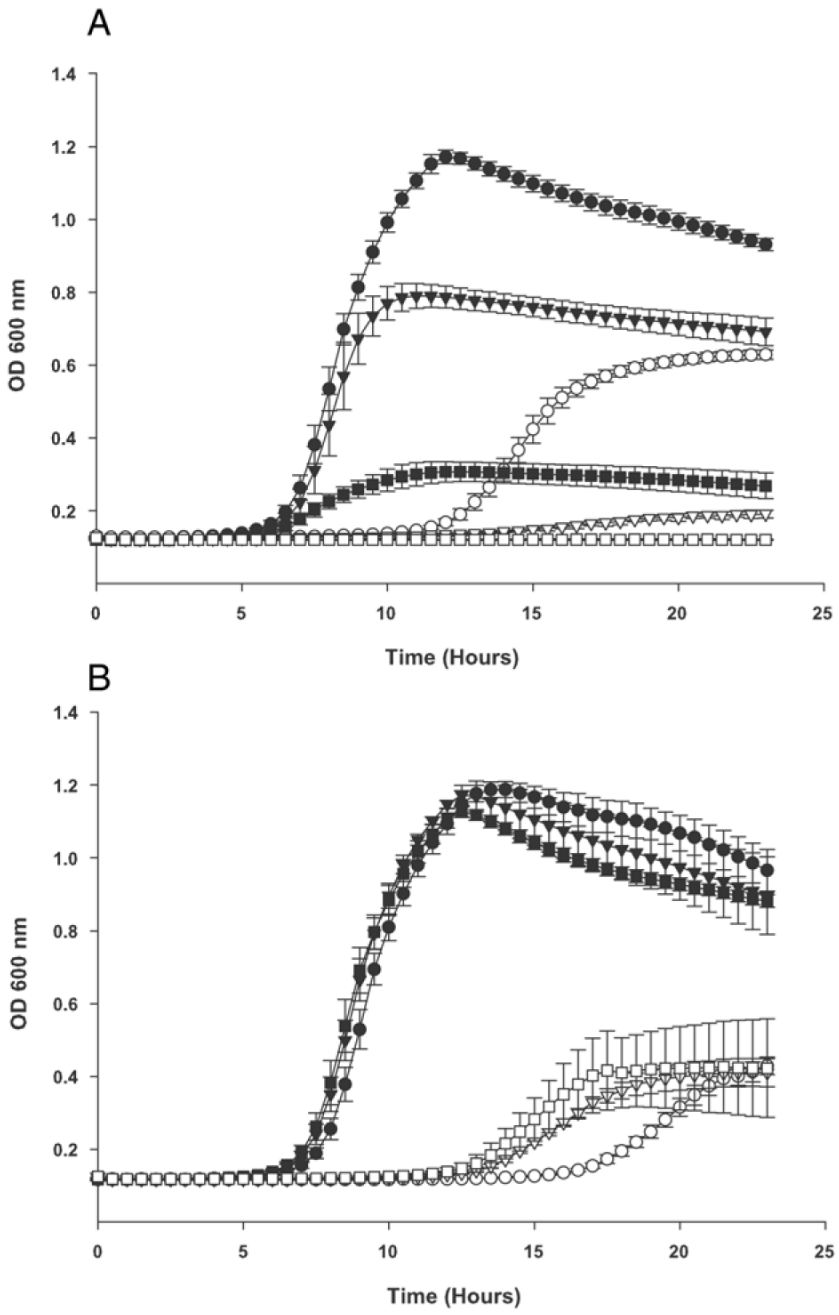

Fig. 1.

A) Growth of the *H. influenzae* nontypeable strain 86-028NP and the *hbpA* insertion mutant strain HI2092 in hdBHI supplemented with heme as the sole heme source. Wild-type strain 86-028NP at 2 μg ml^{-1} heme (solid circles), at 1 μg ml^{-1} (solid triangles), and at 500 ng ml^{-1} (solid squares). The *hbpA* insertion mutant strain HI12092 at 2 μg ml^{-1} heme (open circles), at 1 μg ml^{-1} (open triangles), and at 500 ng ml^{-1} (open squares). B) Growth of the *H. influenzae* nontypeable strain 86-028NP and the *hbpA* insertion mutant strain HI2092 in hdBHI supplemented with hemoglobin, hemoglobin–haptoglobin, or heme–human serum albumin complexes as the sole heme source. Wild-type strain 86-028NP in heme–albumin 200 ng ml^{-1} heme equivalent (solid circles), in hemoglobin 10 μg ml^{-1} (solid squares), and in

hemoglobin–haptoglobin 10 μg ml^{-1} hemoglobin equivalent (solid triangles). The *hbpA* insertion mutant strain HI2092 in heme–albumin 200 ng ml^{-1} heme equivalent (open circles), in hemoglobin 10 μg ml^{-1} (open squares), and in hemoglobin–haptoglobin 10 μg ml^{-1} hemoglobin equivalent (open triangles). Results are mean ± SD for quintuplicate results from representative experiments. The Kruskal-Wallis test was used to compare growth of 86-028NP and HI2092 in all heme sources at all heme concentrations $p<0.0001$.

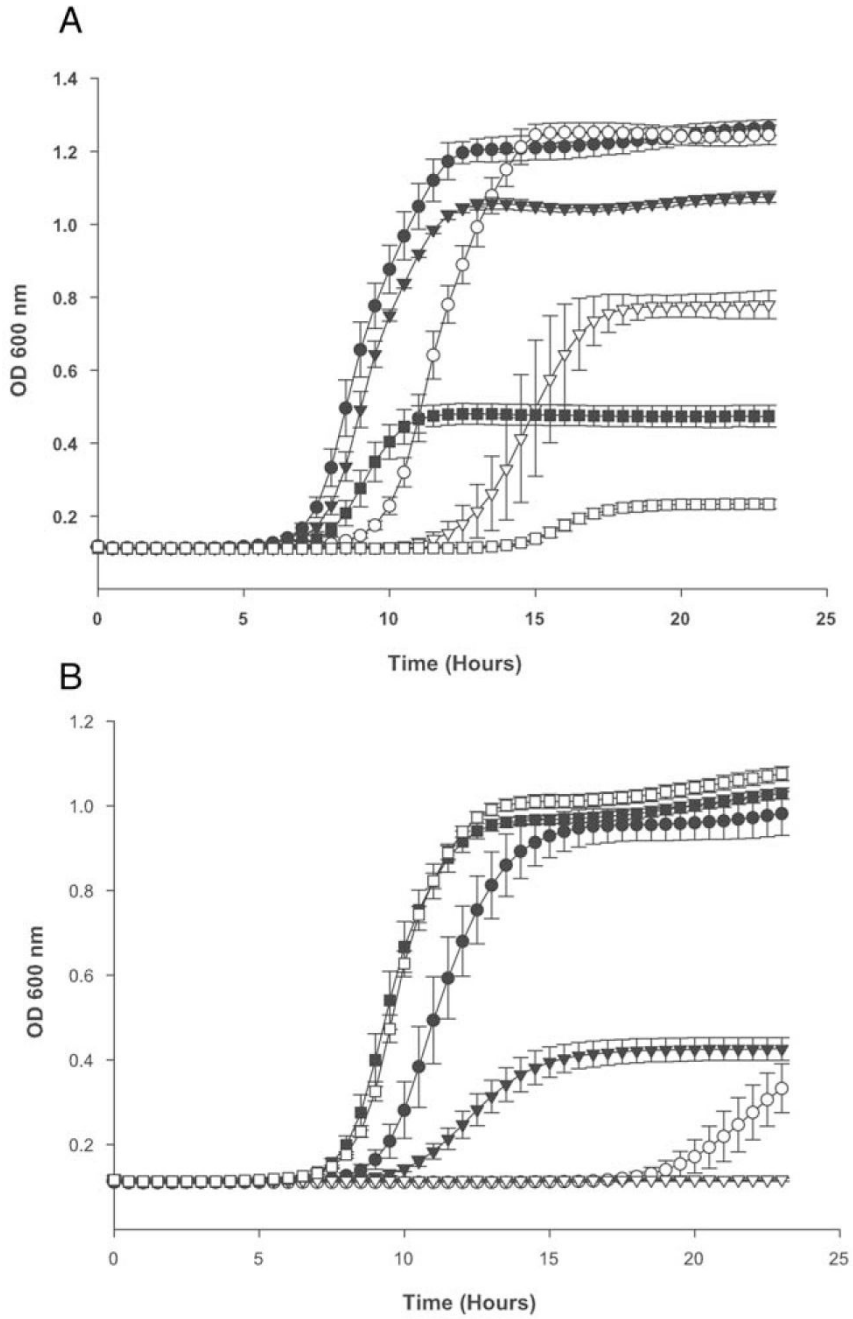

Fig. 2.

A) Growth of the *H. influenzae* type b strain HI689 and the *hbpA* insertion mutant strain HI1886 in hdBHI supplemented with heme–human serum albumin complexes as the sole heme source. Wild-type strain HI689 in heme–albumin 100 ng ml^{-1} heme equivalent (solid circles), in heme–albumin 50 ng ml^{-1} heme equivalent (solid triangles), and in heme–albumin 25 ng ml^{-1} heme equivalent (solid squares). The *hbpA* insertion mutant strain HI1886 in heme–albumin 100 ng ml^{-1} heme equivalent (open circles), in heme–albumin 50 ng ml^{-1} heme equivalent (open triangles), and in heme–albumin 25 ng ml^{-1} heme equivalent (open squares). B) Growth of the *H. influenzae* type b strain HI689 and the *hbpA* insertion mutant strain HI1886 in hdBHI supplemented with heme–hemopexin complexes or hemoglobin–haptoglobin complexes as the

sole heme source. Wild-type strain HI689 in heme–hemopexin 10 μg ml^{-1} heme equivalent (solid circles) and in heme–hemopexin 5 μg ml^{-1} heme equivalent (solid triangles). The *hbpA* insertion mutant strain HI1886 in heme–hemopexin 10 μg ml^{-1} heme equivalent (open circles) and in heme–hemopexin 5 μg ml^{-1} heme equivalent (open triangles). Wild-type strain HI689 in hemoglobin–haptoglobin 2 μg ml^{-1} hemoglobin equivalent (solid squares). The *hbpA* insertion mutant strain HI1886 in hemoglobin–haptoglobin 2 μg ml^{-1} hemoglobin equivalent (open squares). Results are mean ± SD for quintuplicate results from representative experiments. Using the Kruskal-Wallis test to compare growth of HI689 and HI1886 in heme-albumin at 100 ng ml^{-1} heme equivalent $P=0.4156$ over the entire growth curve and $P=0.0036$ for growth between 6 hours and 18 hour; at all other concentrations of heme-albumin $P<0.0001$. The Kruskal-Wallis test was used to compare growth of HI689 and HI1886 in heme–hemopexin at all other concentrations $p<0.0001$. The Kruskal-Wallis test was used to compare growth of HI689 and HI1886 in hemoglobin–haptoglobin at 2 μg ml^{-1} hemoglobin equivalent $p=0.3213$.

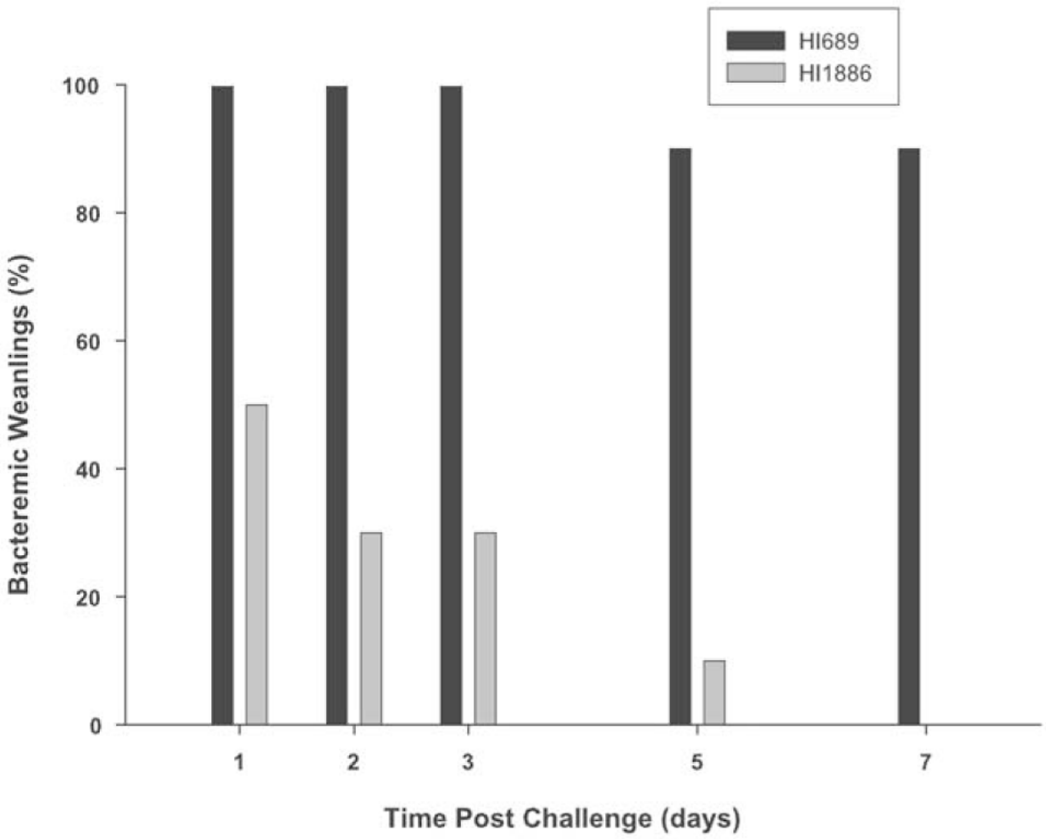

Fig. 3.

Percentage of rats with detectable bacteremia in cohorts of 30-day old weanling rats infected with either the wild-type *H. influenzae* type b strain HI689 (white bars) or the corresponding *hbpA* mutant strain HI1886 (black bars). Differences are statistically significant on days 1, 2, 3, 5, and 7 post-infection ($p<0.02$ on day 1, $p<0.0003$ on days 2, 3, and 5, $p<0.0001$ on day 7).