Published in final edited form as:

Microbes Infect. 2007 July ; 9(8): 932–939.

Lipoprotein e (P4) of *Haemophilus influenzae*: Role in heme utilization and pathogenesis

Daniel J. Morton^a, Ann Smith^b, Timothy M. VanWagoner^a, Thomas W. Seale^a, Paul W. Whitby^a, and Terrence L. Stull^{a,c,*}

^a Department of Pediatrics, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104

^c Department of Microbiology/Immunology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104

^b School of Biological Sciences, University of Missouri-Kansas City, Kansas City, Missouri 64110

Abstract

Lipoprotein *e* (P4) of *Haemophilus influenzae* is a phosphomonoesterase, encoded by the *hel* gene, that has been implicated in the acquisition of heme by this fastidious organism. However, lipoprotein *e* (P4) is also involved in the utilization of NAD and NMN. Some reports have concluded that the reported heme-related growth defect actually reflects a growth defect for NAD. In the current study *hel* insertion mutants were constructed and a role for *e* (P4) in heme acquisition was demonstrated independent of its role in NAD or NMN acquisition. In addition a rat model of infection demonstrated a role for *e* (P4) in the pathogenesis of invasive disease.

Keywords

HAEMOPHILUS; IRON; HEME; VIRULENCE

1. Introduction

Since *H. influenzae* is unable to synthesize the porphyrin ring, it has an absolute requirement for a source of protoporphyrin IX (PPIX) or heme [1]. In the human host heme sources are largely intracellular as hemoglobin or heme-containing enzymes. Free hemoglobin is bound by the serum protein haptoglobin, and the complex is rapidly cleared from the circulation. Free heme is bound by the serum proteins hemopexin and albumin and cleared from the circulation. Hemoglobin, hemoglobin-haptoglobin, heme-hemopexin, and heme-albumin can be utilized by *H. influenzae* *in vitro*; *H. influenzae* has evolved a complex array of uptake mechanisms to utilize available porphyrin [2].

One proposed component of the heme-acquisition pathway(s) is lipoprotein *e* (P4) encoded by the *hel* gene [3]; Reidl and Mekalanos reported that a *hel* mutant exhibited reduced utilization of heme or hemoglobin under aerobic conditions [3]. Reilly *et al.* subsequently identified *e* (P4) as a phosphomonoesterase [4,5], while Reidl *et al.* reported that *e* (P4) has NADP

* Corresponding author Mailing address: Department of Pediatrics, CHO 2308, 940 NE 13th St, Oklahoma City, OK, 73104, Phone: (405)271-4401, Fax: (405)271-8710, E-mail: terrence-stull@ouhsc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

phosphatase activity and is required for utilization of NAD and NADP [6]. Reidl *et al.* concluded that the report with respect to heme utilization was erroneous, stating “it seems unlikely that *e* (P4) is involved in haem uptake” [6]. The fact that growth studies in the original report were performed on media containing NAD [3] supports this view, since *e* (P4) is necessary for utilization of NAD [3,7]; *H. influenzae* lacks the enzymes for de novo synthesis of NAD, thus having an absolute requirement for this nutrient [8]. However, this conclusion does not adequately address the observation that *e* (P4) complements an *E. coli hemA* mutant for growth on heme (the *E. coli hemA* mutant is unable to synthesis heme de novo and the *E. coli* outer membrane is impermeable to heme) [3]. Thus, questions remain regarding the potential role of *e* (P4) in heme acquisition by. This study aimed to clarify whether *e* (P4) has a role in heme acquisition and to assess the impact of *e* (P4) in virulence.

2. Materials and methods

2.1 Bacterial strains, growth conditions and plasmids

Strains and plasmids are listed in Table 1. *H. influenzae* were maintained on chocolate agar with bacitracin at 37°C. When necessary, *H. influenzae* were grown on brain heart infusion (BHI) agar containing 10 µg/ml heme and 10 µg/ml β-NAD (supplemented BHI; sBHI) and the appropriate antibiotic(s). The *hel* mutants were grown on BHI containing 10 µg/ml heme and 10 µg/ml β-nicotinamide mononucleotide (sBHI/NMN) and the appropriate antibiotic(s). Heme-deplete growth was performed in BHI containing 10 µg/ml β-NMN only (heme-deplete BHI; hdBHI/NMN). β-NAD and β-NMN were obtained from Sigma.

2.2 Heme sources

Human hemoglobin, human haptoglobin, human serum albumin (HSA), and hemin were purchased from Sigma. Stock solutions were prepared as previously described [9,10]. Rabbit hemopexin was prepared as described previously [11].

2.3 Construction of *hel* mutants

Insertional mutation of *hel* was achieved as follows. Primers were designed to amplify an 873-bp region encompassing the *hel* gene. Primers were designated eP4-1 (5'-ATACCCTGAATGAATAGG-3') and eP4-2 (5'-AAATCGATCTTTTAAATGG-3'). PCRs were performed as previously described [11] using an annealing temperature of 51°C. Products were successfully cloned into the TA cloning vector pCR2.1-TOPO and confirmed by DNA sequencing. A plasmid harboring the correct insert was designated pSG5. The erythromycin resistance marker from pERM_R was excised with *Sma*I and cloned into the unique *Sna*BI site (internal to *hel*) of pSG5 to yield pSG17. *H. influenzae* were transformed to erythromycin resistance with pSG17 as previously described [12]. Correct chromosomal recombinations were confirmed by the molecular size of a PCR product resolved on an agarose gel.

2.4 Complementation of mutants

A 1255-bp PCR product, encompassing the entire *hel* coding sequence, plus 276-bp upstream of the start codon and 154-bp downstream of the stop codon, was amplified using primers eP4-5 (5'-GGATCCCCCTTAGTTTAAATTTTGCG-3') and eP4-6 (5'-CTGCAGGGCTTGCAACATCATAGC-3') (annealing at 52°C). The primers added a *Bam*HI and a *Pst*I site to the ends of the amplicon. PCR products were cloned into pCR2.1-TOPO to yield pDJM348 and confirmed by DNA sequencing. The insert of pDJM348 was excised with *Bam*HI and *Pst*I and ligated to *Bam*HI/*Pst*I digested pSU2718 to yield pDJM350. pDJM350 was electroporated into *hel* deletion strains.

2.5 Growth Studies

Growth studies were performed using the Bioscreen C Microbiology Reader (Oy Growth Curves AB Ltd., Helsinki, Finland) as previously described [13].

2.6 Animal Model

A rat model was used to assess bacteremia in both 5-day old and 30-day old rats as previously described [10,11,14]. Specified pathogen free timed-pregnant Sprague-Dawley rats were received five days prior to parturition. Newborn pups from different mothers were pooled and randomly reassigned to the mothers (n=10 pups per female). Pups were weaned at 21 days. Five-day old rats were inoculated with 200 cfu, and 30-day old rats were inoculated with 2000 cfu by intraperitoneal injection. Animal protocols were approved by the Institutional Animal Use and Care Committee of the University of Oklahoma Health Sciences Center.

2.7 Statistics

Statistical comparisons of growth *in vitro* and in infected animals were made using the Kruskal-Wallis test (Analyse-It for Microsoft Excel v1.71). A *P* value < 0.01 was taken as statistically significant.

Bacteremic titers are expressed as mean values $\pm$ SD typically from groups of 10 animals. Percentages of bacteremic pups were compared by the Fisher Exact Test performed with SigmaStat software. A *P* value < 0.05 was taken as statistically significant.

3. Results

3.1 Growth characteristics

To investigate the role of *e* (P4) in heme utilization we constructed *hel* insertion mutations in strains HI689 and Rd KW20. Mutant strains did not grow on sBHI agar (data not shown); however, both mutant strains grew as well as the wild type on sBHI/NMN. These findings indicate that while *e* (P4) is essential for utilization of NAD (10 μ g/ml; 15 μ M), it is not necessary for the utilization of NMN (10 μ g/ml; 30 μ M). These data confirm the report of Kemmer *et al.* that an *e* (P4) mutant did not grow on media containing NAD below 35 μ M but grew on 15 μ M NMN [7]. Therefore, media in subsequent growth studies was supplemented with NMN at 10 μ g/ml.

Comparison of strain HI689 and the *hel* mutation derivative HI1889 demonstrated no significant growth difference at high levels of heme (Figure 1). Growth of HI689 and HI1889 was comparable at 20 μ g/ml heme (Figure 1, Panel A: *P*=0.04 over the entire curve and *P*=0.16 over the first 20 hours), and at 10 μ g/ml heme (Figure 1, Panel B: *P*=0.09); however, at 10 μ g/ml there was delayed onset of growth of HI1889. At lower heme levels the mutant strain grew significantly less well than the wild type strain. For example, at 1 μ g/ml HI1889 exhibited both a delayed onset of growth and a lower final OD than HI689 (Figure 1, Panel C: *P*<0.0001). Complementation of the *hel* mutation *in trans* restored growth of the mutant strain to wild type levels (Figure 1, Panel C: *P*=0.07 comparing HI689 and HI1944), while strain HI1889 carrying the vector pSU2718 grew similarly to the mutant strain (Figure 1, Panel C). We also compared strain Rd KW20 and its *hel* insertion mutant strain HI1893. No significant differences was observed between these strains at 10 μ g/ml heme (*P*=0.36). However, at 5 μ g/ml and 2 μ g/ml heme growth was delayed in onset and reached a lower final OD (*P*<0.0001 for both growth conditions). These data demonstrate a specific role for *e* (P4) in the acquisition of heme in two *H. influenzae* strains.

Utilization of hemoglobin, hemoglobin-haptoglobin, heme-albumin and heme-hemopexin was also examined, and all gave results comparable to those obtained with heme. However, in some

cases strains HI689 and Rd KW20 varied with respect to levels of the heme source at which the impact of the *hel* mutation became apparent. At a hemoglobin concentration of 10 µg/ml there was no significant difference between HI689 and HI1889 (Figure 2, Panel A: $P=0.02$). In contrast Rd KW20 grew significantly better than its corresponding mutant at the same hemoglobin concentration (Figure 2, Panel B: $P<0.0001$). At 5 µg/ml hemoglobin, the *hel* mutants of both strains grew less well than the wild type strains ($P<0.0001$ for both strains).

At a hemoglobin-haptoglobin concentration equivalent to 20 µg/ml hemoglobin, HI1889 exhibited a significantly delayed onset of growth compared to HI689, although it attained the same final OD, (Figure 3, Panel A: $P<0.0001$). At lower concentrations of hemoglobin-haptoglobin growth of the mutant strain was both delayed in onset and attained a lower final OD than the wild type ($P<0.0001$ for growth at hemoglobin-haptoglobin concentrations equivalent to 10 µg/ml, 5 µg/ml and 2.5 µg/ml hemoglobin). Panel B of Figure 3 shows a representative experiment with 5 µg/ml hemoglobin as hemoglobin-haptoglobin. Complementation of the mutant strain HI1889 partially restored growth (Figure 3, Panel B). In contrast growth of HI1889 harboring the vector pSU2718 was indistinguishable from the mutant strain (Figure 3, Panel B: $P=0.64$). Comparison of Rd KW20 and its mutant derivative in hemoglobin-haptoglobin yielded comparable results.

Growth of the *hel* insertion mutants with heme serum albumin (100 ng/ml with respect to heme) exhibited delayed onset of growth, a reduced growth rate and a lower final OD compared to the wild type strains ($P<0.0001$ for HI689 versus HI1889 and for Rd KW20 versus HI1893). Complementation reversed the growth defect in the mutant strains. Growth of *hel* mutant strains with the heme-hemopexin complex at 2 µg/ml or 1 µg/ml gave similar results. In Figure 4 growth of both HI689 (Panel A) and Rd KW20 (Panel B) is compared to their respective mutants at 2 µg/ml heme-hemopexin ($P<0.0001$ for both strain pairs). Thus, regardless of the heme source, *e* (P4) mutants were impaired in their ability to utilize heme.

3.2 Contribution of *hel* to virulence in the rat model of invasive disease

The effect of the *hel* mutation on virulence was assessed in rats infected at either 5 or 30 days of age with strain HI689 or its *hel* mutant derivative HI1889. In 5-day old rats both strains were equally able to establish infection. All rat pups infected with either strain HI689 ($n=10$) or HI1889 ($n=10$) exhibited bacteremia within 24 hours. Bacteremia persisted in all rat pups for the seven days of the assessment period. Maximal bacteremic titers achieved by the two strains were comparable ($1.7 \times 10^5 \pm 2.9 \times 10^5$ and $1.5 \times 10^5 \pm 2.0 \times 10^5$ c.f.u./ml respectively for HI689 and HI1889). There was a trend toward lower bacteremic titers for the first two days following infection with HI1889 (Figure 5), however the bacteremic titer curves for the two strains showed no overall statistically significant difference.

In 30-day old rats the wild type strain more readily established infection than did the mutant strain (Figure 5). On day one, 8 of 10 rats infected with HI689 were bacteremic while 2 of 10 rats infected with HI1889 had detectable bacteremia ($P<0.03$). By day three all rats infected with HI1889 had cleared the bacteria while 50% of the HI689 infected rats remained bacteremic ($P<0.01$). The bacteremic titer curves for the two strains demonstrated that strain HI1889 yielded significantly lower bacteremic titers than did HI689; the bacteremic titer for strain HI1889 on day 2 reflects an atypically high titer from a single infected rat (Figure 5, Panel B).

4. Discussion

This study aimed to establish whether *e* (P4) is involved in heme utilization *in vitro* and infection in an animal model. The first report that *e* (P4) is involved in heme utilization was based on the ability of cloned *e* (P4) to complement an *E. coli hemA* mutant for growth on heme, and the apparently reduced ability of a *hel* insertion mutant to grow on media

supplemented with NAD and either heme or hemoglobin [3]. Subsequent studies demonstrated that *e* (P4) is involved in the utilization of NAD and NADP and led the authors to “argue that the original observed aerobic growth defect of *hel* mutant strains is based on a deficiency in using factor V and not haemin” [6]. Such a conclusion seems unlikely since 1) it fails to explain the ability of cloned *hel* to complement a *hemA* mutant of *E. coli* for growth on heme and 2) it does not address the fact that there was no reported growth defect for the *hel* insertion mutants when grown anaerobically. Under anaerobic conditions there is no requirement for heme while the presence of NAD is essential. We have successfully subcultured four *H. influenzae* strains for up to 20 generations under anaerobic conditions on a chemically defined medium without heme, but with NAD. However, on the same medium with heme and without NAD the strains do not grow even on the first subculture under anaerobic conditions (unreported observation). Thus, if the phenotype of the *hel* mutant were due to a defect in utilization of NAD it should be evident under anaerobic conditions as well as aerobic conditions. To address the question of the role of *e* (P4) in heme utilization, we eliminated the complicating factor of NAD utilization by performing growth studies with NMN to satisfy the factor V requirement. Although *e* (P4) has also been implicated in utilization of NMN there is no discernible impact of a *hel* insertion mutation on utilization of high levels of NMN in either a prior report or in our laboratory [7]. At high levels of free heme (20 µg/ml) no difference in growth was seen between the wild type and *hel* mutant strains. However, at lower heme levels the *hel* insertion mutants grew significantly less well than the wild type strain and the growth defect was reversed by complementation with *hel* in trans. The *hel* mutant was also deficient in its ability to utilize protein bound heme sources expected to be present in vivo. The growth phenotype cannot be attributed to a defect in NMN utilization since one would expect to see the reduced growth at all heme concentrations if this were the case. Since an *e* (P4) mutation has no impact on utilization of nicotinamide riboside (NR) [7], in some growth studies NMN was replaced with NR generated from NMN as previously described [15]. Growth studies with NR gave similar results to those utilizing NMN (data not shown). These data indicate that *e* (P4) is a component of a high affinity heme acquisition pathway in *H. influenzae*.

Lipoprotein *e* (P4) has been identified as a member of a enzyme family termed the DDDD phosphohydrolases [16–18]. All members of this family are characterized by two pairs of conserved aspartate residues located in different regions of the protein and separated by a variable spacer region [17,18]. More specifically *e* (P4) falls into a subfamily of the DDDD phosphohydrolases termed the bacterial class C nonspecific acid phosphohydrolases (NSAPs) [18]. The phosphohydrolase activity of *e* (P4) can be separated from its role in heme utilization. Mutation of any one of the four conserved aspartate residues in *e* (P4) abolishes the phosphomonoesterase activity [16]. Lipoprotein *e* (P4) with either the D181N or the D185N mutation was able to complement a *hemA* mutant of *E. coli* for growth on heme, while the D64A and D66A mutation derivatives could not do so [16]. The authors speculated that the impact of the D64A and D66A mutations resulted from proximity to a putative heme-binding site proposed by Reidl and Mekalanos and located at K45-H50 [3,16]. Additionally D64 and D66 themselves are unlikely to be required for the heme utilization function since a fusion protein maintaining the coding sequence of only the first 49 amino acids of the mature protein was able to complement the *hemA* mutation [3]. The putative heme-binding site (KVAFDH) was identified based on homology to regions of known heme-binding proteins [3]. A mutation in the heme-binding site (F48C) abolished phosphomonoesterase activity and rendered the protein unable to complement the *E. coli hemA* mutant for growth on heme [16]. In addition, a fusion protein retaining the coding sequence for the first 30 amino acids of the mature *e* (P4), thus lacking the heme-binding site, was unable to complement the *hemA* mutation in *E. coli* [3]. Point mutations in either the conserved aspartate residues or the proposed heme binding site have not been analyzed in *H. influenzae* for an impact on heme acquisition; however, point mutations of D64 and D66 in *H. influenzae* reportedly impacted utilization of NAD [7]. Other members of the bacterial class C NSAP family that have been partially characterized include

HppA of *Helicobacter pylori*, OlpA of *Chryseobacterium meningosepticum*, LppC of *Streptococcus equisimilis*, and SapS of *Staphylococcus aureus* [19–23]. Of these only LppC was analyzed with respect to heme utilization and is unable to complement the *hemA* mutant of *E. coli* [21]. LppC does not contain a region homologous to the putative heme binding site of *e* (P4); *S. equisimilis* is neither able to bind nor utilize heme as an iron source [21]. OlpA similarly contains no region homologous to the heme-binding site and we could find no reports on the ability of *C. meningosepticum* to utilize heme. However, other members of the protein family, including HppA and SapS, contain regions of significant homology to the putative heme-binding region. The potential role of the class C NSAPs of these organisms in the acquisition of heme may warrant investigation.

The animal studies reported here are the first to demonstrate a role for *e* (P4) in invasive disease. Virulence was attenuated, as judged by reduced bacteremic titers and more rapid clearance of infection, in the *hel* insertion mutant strain compared to its wild type progenitor in 30-day old rats. The effect appeared to be age dependent since 5-day old rats exhibited only a trend towards diminished titers and no difference in clearance when infected with the mutant strain. This finding may reflect differences in the predominant heme source available to in 5-day old and 30-day old rats. It is well established in humans that significant changes in serum concentrations of heme related proteins occur during infant development. Hemopexin serum levels increase significantly between day one postpartum and 6 months of age; in term infants 95th percentile serum hemopexin concentrations increased from 0.44 g/l at 1 day to 0.78 g/l at 1 month, 0.92 g/l at 2 months and reached adult levels of 1.11 g/l at 6 months [24]. Haptoglobin was undetectable in the serum of 60% of term infants on day one postpartum; by 6 months of age haptoglobin could be detected in all infants with 95th percentile serum concentrations increasing from 1.08 g/l at 1 month to 1.65 g/l at 2 months and 3.24 g/l at 6 months [24]. Serum albumin levels also increase with age, although less significantly than either hemopexin or haptoglobin levels [24]. Similar changes in these serum proteins occur in the first month of life in infant rats [25]. While we cannot definitively identify the predominant heme source(s) available in either 5-day old or 30-day old rats, the data referenced above indicates an increasing availability of both hemoglobin-haptoglobin and heme-hemopexin in the developing rat. It remains possible that there is an, as yet unidentified, additional heme source available in the 5-day old rat that becomes unavailable as the rat ages. We have seen similar age-related differences in virulence when comparing strain HI689 with a mutant derivative lacking the hemoglobin-haptoglobin binding proteins [14]. These proteins are essential for the utilization of hemoglobin-haptoglobin [9] and the age-related difference in virulence could be explained by hemoglobin-haptoglobin complexes becoming the predominant available heme source in 30-day old rats [14]. We have also recently reported age-related differences in virulence when comparing a type b strain with a mutant in the heme-hemopexin utilization genes (*hxuCBA*) [10].

Since *e* (P4) is also known to be involved in utilization of NAD and NMN, the difference in response to invasive infection between the two age groups of rats could be due to variation in levels of this essential nutrient. However, this explanation seems unlikely; NAD is of paramount importance in the physiology of the cell and its levels are maintained [26]. There is also significant NAD turnover in human blood and both NMN and NR can be detected [27]. NR can satisfy the factor V requirement of *H. influenzae*, and *e* (P4) mutants are unaffected in their ability to use it [7]. Schmidt-Brauns *et al.* identified NAD and NMN nucleotidase activities in the blood of seven-day old infant rats and concluded that these enzymatic activities would provide adequate NR levels to satisfy the factor V requirement during invasive disease [28].

In conclusion *e* (P4) is a component of a high affinity heme acquisition pathway as well as having an established role in factor V utilization. The precise role of *e* (P4) in heme acquisition

is not yet clear, and elucidation of its role is complicated by the multiplicity of heme acquisition mechanisms in *H. influenzae* [2]. Future work should assess the precise role of *e* (P4) in heme utilization and elucidate its potential interactions with other proteins involved in acquisition of this essential nutrient.

Acknowledgements

This work was supported by Public Health Service Grant AI29611 from the National Institutes of Allergy and Infectious Diseases. We acknowledge the support of the Children's Medical Research Institute.

References

1. Evans NM, Smith DD, Wicken AJ. Haemin and nicotinamide adenine dinucleotide requirements of *Haemophilus influenzae* and *Haemophilus parainfluenzae*. *J Med Microbiol* 1974;7:359–365. [PubMed: 4371115]
2. Morton, DJ.; Stull, TL. *Haemophilus*. In: Crosa, JH.; Mey, AR.; Payne, SM., editors. *Iron Transport in Bacteria*. American Society for Microbiology; Washington, DC: 2004. p. 273–292.
3. Reidl J, Mekalanos JJ. Lipoprotein *e*(P4) is essential for hemin uptake by *Haemophilus influenzae*. *J Exp Med* 1996;183:621–629. [PubMed: 8627173]
4. Reilly TJ, Chance DL, Smith AL. Outer membrane lipoprotein *e* (P4) of *Haemophilus influenzae* is a novel phosphomonoesterase. *J Bacteriol* 1999;181:6797–6805. [PubMed: 10542183]
5. Reilly TJ, Smith AL. Purification and characterization of a recombinant *Haemophilus influenzae* outer membrane phosphomonoesterase *e* (P4). *Protein Expr Purif* 1999;17:401–409. [PubMed: 10600458]
6. Reidl J, Schlor S, Kraiss A, Schmidt-Brauns J, Kemmer G, Soleva E. NADP and NAD utilization in *Haemophilus influenzae*. *Mol Microbiol* 2000;35:1573–1581. [PubMed: 10760156]
7. Kemmer G, Reilly TJ, Schmidt-Brauns J, Zlotnik GW, Green BA, Fiske MJ, Herbert M, Kraiss A, Schlor S, Smith A, Reidl J. NadN and *e*(P4) are essential for utilization of NAD and nicotinamide mononucleotide but not nicotinamide riboside in *Haemophilus influenzae*. *J Bacteriol* 2001;183:3974–3981. [PubMed: 11395461]
8. Gerlach G, Reidl J. NAD⁺ utilization in *Pasteurellaceae*: simplification of a complex pathway. *J Bacteriol* 2006;188:6719–6727. [PubMed: 16980474]
9. Morton DJ, Whitby PW, Jin H, Ren Z, Stull TL. Effect of multiple mutations in the hemoglobin- and hemoglobin-haptoglobin-binding proteins, HgpA, HgpB, and HgpC of *Haemophilus influenzae* type b. *Infect Immun* 1999;67:2729–2739. [PubMed: 10338475]
10. Morton DJ, Seale TW, Madore LL, VanWagoner TM, Whitby PW, Stull TL. The haem-haemopexin utilization gene cluster (*hxcBA*) as a virulence factor of *Haemophilus influenzae*. *Microbiology* 2007;153:216–225.
11. Morton DJ, Smith A, Madore LL, VanWagoner TM, Seale TW, Whitby PW, Stull TL. Identification of a haem utilization protein (Hup) in *Haemophilus influenzae*. *Microbiology* 2004;150:3923–3933. [PubMed: 15583146]
12. Morton DJ, Bakaletz LO, Jurcisek JA, VanWagoner TM, Seale TW, Whitby PW, Stull TL. Reduced severity of middle ear infection caused by nontypeable *Haemophilus influenzae* lacking the hemoglobin/hemoglobin-haptoglobin binding proteins (Hgp) in a chinchilla model of otitis media. *Microb Pathog* 2004;36:25–33. [PubMed: 14643637]
13. Morton DJ, Madore LL, Smith A, VanWagoner TM, Seale TW, Whitby PW, Stull TL. The heme-binding lipoprotein (HbpA) of *Haemophilus influenzae*: role in heme utilization. *FEMS Microbiol Lett* 2005;253:193–199. [PubMed: 16289530]
14. Seale TW, Morton DJ, Whitby PW, Wolf R, Kosanke SD, VanWagoner TM, Stull TL. Complex role of hemoglobin and hemoglobin-haptoglobin binding proteins in *Haemophilus influenzae* virulence in the infant rat model of invasive infection. *Infect Immun* 2006;74:6213–6225. [PubMed: 16966415]
15. Bieganowski P, Brenner C. Discoveries of nicotinamide riboside as a nutrient and conserved NRK genes establish a Preiss-Handler independent route to NAD⁺ in fungi and humans. *Cell* 2004;117:495–502. [PubMed: 15137942]

16. Reilly TJ, Green BA, Zlotnick GW, Smith AL. Contribution of the DDDD motif of *H. influenzae* e (P4) to phosphomonoesterase activity and heme transport. *FEBS Lett* 2001;494:19–23. [PubMed: 11297727]
17. Rossolini GM, Schippa S, Riccio ML, Berlutti F, Macaskie LE, Thaller MC. Bacterial nonspecific acid phosphohydrolases: physiology, evolution and use as tools in microbial biotechnology. *Cell Mol Life Sci* 1998;54:833–850. [PubMed: 9760992]
18. Thaller MC, Schippa S, Rossolini GM. Conserved sequence motifs among bacterial, eukaryotic, and archaeal phosphatases that define a new phosphohydrolase superfamily. *Protein Sci* 1998;7:1647–1652. [PubMed: 9684901]
19. Godlewska R, Bujnicki JM, Ostrowski J, Jagusztyn-Krynicka EK. The *hpaA* gene of *Helicobacter pylori* encodes the class C acid phosphatase precursor. *FEBS Lett* 2002;525:39–42. [PubMed: 12163158]
20. du Plessis EM, Theron J, Joubert L, Lotter T, Watson TG. Characterization of a phosphatase secreted by *Staphylococcus aureus* strain 154, a new member of the bacterial class C family of nonspecific acid phosphatases. *Syst Appl Microbiol* 2002;25:21–30. [PubMed: 12086184]
21. Gase K, Liu G, Bruckmann A, Steiner K, Ozegowski J, Malke H. The *lppC* gene of *Streptococcus equisimilis* encodes a lipoprotein that is homologous to the e (P4) outer membrane protein from *Haemophilus influenzae*. *Med Microbiol Immunol (Berl)* 1997;186:63–73. [PubMed: 9255768]
22. Malke H. Cytoplasmic membrane lipoprotein LppC of *Streptococcus equisimilis* functions as an acid phosphatase. *Appl Environ Microbiol* 1998;64:2439–2442. [PubMed: 9647812]
23. Passariello C, Schippa S, Iori P, Berlutti F, Thaller MC, Rossolini GM. The molecular class C acid phosphatase of *Chryseobacterium meningosepticum* (OlpA) is a broad-spectrum nucleotidase with preferential activity on 5'-nucleotides. *Biochim Biophys Acta* 2003;1648:203–209. [PubMed: 12758163]
24. Kanakoudi F, Drossou V, Tzimouli V, Diamanti E, Konstantinidis T, Germentis A, Kremenopoulos G. Serum concentrations of 10 acute-phase proteins in healthy term and preterm infants from birth to age 6 months. *Clin Chem* 1995;41:605–608. [PubMed: 7536645]
25. Moldenhauer H, Rose H. Entwicklung der serumeiweisse von ratten im ersten monat nach der geburt. *Acta Biol Med Ger* 1970;25:469–472. [PubMed: 4108526]
26. Magni G, Amici A, Emanuelli M, Orsomando G, Raffaelli N, Ruggieri S. Enzymology of NAD+ homeostasis in man. *Cell Mol Life Sci* 2004;61:19–34. [PubMed: 14704851]
27. Friedlos F, Knox RJ. Metabolism of NAD(P)H by blood components. Relevance to bioreductively activated prodrugs in a targeted enzyme therapy system. *Biochem Pharmacol* 1992;44:631–635. [PubMed: 1387314]
28. Schmidt-Brauns J, Herbert M, Kemmer G, Kraiss A, Schlör S, Reidl J. Is a NAD pyrophosphatase activity necessary for *Haemophilus influenzae* type b multiplication in the blood stream? *Int J Med Microbiol* 2001;291:219–225. [PubMed: 11554562]
29. Musser JM, Barenkamp SJ, Granoff DM, Selander RK. Genetic relationships of serologically nontypable and serotype b strains of *Haemophilus influenzae*. *Infect Immun* 1986;52:183–191. [PubMed: 3485574]

Fig. 1. Growth of *H. influenzae* type b strain HI689 (diamonds), the *hel* insertion mutant HI1889 (squares), the *hel* insertion mutant harboring the *hel* bearing plasmid pDJM350 (strain HI1944; triangles) and the *hel* insertion mutant harboring the vector pSU2718 (strain HI1946; ×). Growth is in BHI supplemented with 20 µg/ml heme (Panel A), 10 µg/ml heme (Panel B) or 1 µg/ml heme (Panel C). Results are mean ± SD for quintuplicate results from representative experiments.

Fig. 2.

Growth of *H. influenzae* strains in BHI supplemented with 10 µg/ml hemoglobin. Panel A shows growth of *H. influenzae* type b strain HI689 (solid triangles), and the corresponding *hel* insertion mutant HI1889 (solid squares). Panel B shows growth of *H. influenzae* strain RdKW20 (open triangles), and the corresponding *hel* insertion mutant HI1893 (open squares). Results are mean $\pm$ SD for quintuplicate results from representative experiments.

Fig. 3. Growth of *H. influenzae* type b strain HI689 (diamonds), the *hel* insertion mutant HI1889 (squares), the *hel* insertion mutant harboring the *hel* bearing plasmid pDJM350 (strain HI1944; diamonds) and the *hel* insertion mutant harboring the vector pSU2718 (strain HI1946; ×). Growth is in BHI supplemented with hemoglobin-haptoglobin complex 20 µg/ml hemoglobin equivalent (Panel A) or hemoglobin-haptoglobin 5 µg/ml hemoglobin equivalent (Panel B). Results are mean ± SD for quintuplicate results from representative experiments.

Fig. 4. Growth of *H. influenzae* strains in BHI supplemented with 2 µg/ml heme-hemopexin complex. Panel A shows growth of *H. influenzae* type b strain HI689 (solid diamonds), and the corresponding *hel* insertion mutant HI1889 (solid squares). Panel B shows growth of *H. influenzae* strain RdKW20 (open triangles), and the corresponding *hel* insertion mutant HI1893 (open squares). Results are mean $\pm$ SD for quintuplicate results from representative experiments.

Fig. 5. Bacteremic titers in 5-day old rats (Panel A) and 30-day old rats (Panel B) infected with the wildtype strain HI689 or the *hel* insertion mutant strain HI1889. Results are means $\pm$ SD from groups of 10 rats. Panel C shows % of bacteremic 30-day old rats infected with the wildtype strain HI689 or the *hel* insertion mutant strain HI1889 (* $P < 0.03$, ** $P < 0.01$)

Table 1
Strains and plasmids used in this study

Strain or plasmid	Relevant Characteristic(s) ^a	Reference or source
Strains		
<i>H. influenzae</i>		
HI689	Type b	[29]
Rd KW20	Sequenced type d derivative	ATCC
HI1889	HI689, <i>hel::erm</i> , <i>Erm^r</i>	This study
HI1893	Rd KW20, <i>hel::erm</i> , <i>Erm^r</i>	This study
HI1944	HI1889, pDJM350 <i>Erm^r</i> <i>Cm^r</i>	This study
HI1946	HI1889, pSU2718 <i>Erm^r</i> <i>Cm^r</i>	This study
<i>E. coli</i>		
TOP10	Used for cloning in pCR2.1-TOPO and pUC19N	Invitrogen
Plasmids		
pCR2.1-TOPO	<i>Plac</i> , <i>lacZα</i> , <i>Kan^r</i> <i>Amp^r</i> , <i>ColE1</i> origin, F1 origin, T7 promoter	Invitrogen
pUC19N	pUC19 with a <i>NotI</i> site added at the <i>HindIII</i> end of the polylinker	
pSU2718	<i>E. coli-H. influenzae</i> shuttle vector, <i>LacZα</i> , <i>Cm^r</i> , p15a ori	
pERM ^r	pCR2.1-TOPO carrying an approximately 1300-bp erythromycin resistance cassette bounded by multiple restriction enzyme sites	[10]
pSG5	pCR2.1-TOPO carrying an 874-bp PCR product encompassing the <i>hel</i> coding sequence	This study
pSG17	pSG5 with the ~1300-bp <i>Erm^r</i> marker from pERM ^r inserted at the unique <i>SnaBI</i> site	This study
pDJM348	pCR2.1-TOPO carrying an 1255-bp PCR product encompassing the <i>hel</i> coding sequence	This study
pDJM350	pSU2718 carrying the 1255-bp product from pDJM348	This study

^a*Erm^r*, erythromycin resistance (8 µg/ml erythromycin in *H. influenzae* and 200 µg/ml erythromycin in *E. coli*); *Cm^r*, chloramphenicol resistance (1.5 µg/ml chloramphenicol in *H. influenzae* and 1.5 µg/ml chloramphenicol in *E. coli*); *Kan^r*, kanamycin resistance (50 µg/ml kanamycin in *E. coli*); *Amp^r*, ampicillin resistance (50 µg/ml ampicillin in *E. coli*)