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Tamim, Al-Jubair; Singh, Birendra; Fleury, Christophe; Blom, Anna; Mörgelin, Matthias; Thunnissen, Marjolein; Riesbeck, Kristian

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Haemophilus influenzae stores and distributes hemin by using Protein E

Running Title: Protein E is a hemin-binding outer membrane protein

Tamim Al Jubair[a], Birendra Singh[a], Christophe Fleury[a], Anna M. Blom[b], Matthias Mörgelin[c], Marjolein M. Thunnissen[d] and Kristian Riesbeck[a, *]

[a]Medical Microbiology and [b]Protein Chemistry, Dept. of Laboratory Medicine Malmö, Lund University, SE-205 02 Malmö, Sweden, [c]Section of Clinical and Experimental Infectious Medicine, Dept. of Clinical Sciences, Lund University, SE-221 84 Lund, Sweden, and [d]Dept. of Biochemistry and Structural Biology, Lund University, SE-221 00 Lund, Sweden

[*]Dr. Kristian Riesbeck, Medical Microbiology, Department of Laboratory Medicine Malmö, Lund University, Jan Waldenströms gata 59, SE-205 02 Malmö, Sweden
Tel.: +46 40 338494; fax: +46 40 336234

E-mail: kristian.riesbeck@med.lu.se

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Abstract

The human pathogen *Haemophilus influenzae* causes mainly respiratory tract infections such as acute otitis media in children and exacerbations in patients with chronic obstructive pulmonary disease. We recently revealed the crystal structure of *H. influenzeae* protein E (PE), a multifunctional adhesin that is involved in direct interactions with lung epithelial cells and host proteins. Based upon the PE structure we here suggest a hypothetical binding pocket that is compatible in size with a hemin molecule. An *H. influenzae* mutant devoid of PE bound significantly less hemin in comparison to the PE-expressing wild type counterpart. Accordingly, *E. coli* expressing PE at the surface bound hemin. An interaction between hemin and recombinant soluble PE was also demonstrated by native-PAGE and UV-visible spectrophotometry. Surface plasmon resonance revealed an affinity (K_d) of 1.6×10^{-6} M for the hemin-PE interaction. Importantly, hemin that was bound to PE at the *H. influenzae* surface, was donated to co-cultured luciferase-expressing *H. influenzae* that were starved of hemin. When hemin is bound to PE it thus may serve as a storage pool for *H. influenzae*. To our knowledge this is the first report showing that *H. influenzae* can share hemin via a surface-located outer membrane protein.

Introduction

The Gram-negative *Haemophilus influenzae* is a member of the *Pasteurellaceae* family, and is classified as encapsulated (*H. influenzae* type a to f) and non-typeable *H. influenzae* (NTHi). After introduction of a vaccine against *H. influenzae* type b (Hib) in the 1990s, NTHi are now responsible for the majority of *Haemophilus* infections (Murphy, 2003). NTHi colonizes the mucosa and causes respiratory tract infections such as acute otitis media in children and exacerbations in patients with chronic obstructive pulmonary disease (COPD), but in rare cases also invasive disease, mainly meningitis and sepsis (Murphy et al., 2009; Resman et al., 2011)

H. influenzae has an absolute requirement for heme since it lacks 6 of 7 enzymes in the heme synthetic pathway that consequently leads to an inability to produce protoporphyrin IX (Loeb, 1995). The success of *H. influenzae* colonization thus depends on its ability to acquire protoporphyrin IX or heme from the host (Infante-Rivard and Fernandez, 1993). Since heme is a major source of iron for most bacteria, some species directly extract iron from heme while other transport heme into the cytosol to retrieve iron (Wandersman and Stojiljkovic, 2000). In the human host, iron is present in complex forms bound to high-affinity iron-binding host proteins such as transferrin, lactoferrin, the iron storage protein ferritin, or incorporated into a protoporphyrin ring (Ratledge and Dover, 2000). Free iron is potentially toxic to living cells since it can convert hydrogen peroxide into free radicals that may cause cellular damage. In fact, the free iron concentration is only $\approx 10^{-18}$ M within the human host (Bullen and Ward, 1988). However, freely available iron may occasionally arise during tissue injury, and hence free heme is released from erythrocytes upon intravascular hemolysis and inflammation caused by

polymorphonuclear neutrophils (Aruoma et al., 1988; Evans et al., 1994; Resman et al., 2011; Skaar, 2010).

Although it is well known that *H. influenzae* grows on laboratory culture media containing heme as iron and protoporphyrin IX source, all key components necessary for utilization of heme have not yet been identified. Recent studies of heme/iron acquisition systems in *H. influenzae* have, however, defined several proteins including FbpA (ferric-ion-binding protein A), HbpA (haemoglobin/haemoglobin-haptoglobin binding protein A), SapA (sensitivity to antimicrobial peptides protein A), and finally HxuA (hem–hemopexin binding protein A) (Fournier et al., 2011; Jin et al., 1996; Jin et al., 1999; Khan et al., 2007; Mason et al., 2011). These proteins are located in the periplasm, but HxuA can also be secreted. Lipoprotein P4 has been described as the only outer membrane protein of *H. influenzae* that binds hemin (Reidl and Mekalanos, 1996).

H. influenzae protein E (PE) is a 16 kDa, highly conserved (96.9%–100%), ubiquitous outer membrane lipoprotein that exists in both encapsulated *H. influenzae* and NTHi (Singh et al., 2010). PE was initially described as an adhesin that binds to epithelial cells of different origins including those from COPD patients (Ronander et al., 2009). In addition, PE simultaneously binds to extracellular matrix proteins and plasminogen, interactions that all contribute to bacterial virulence (Barthel et al., 2012; Hallstrom et al., 2009; Hallstrom et al., 2011). We recently crystallized PE and showed that it is a dimer with multiple binding sites for proteins derived from the host (Hallstrom et al., 2011; Singh et al., 2012; Singh et al., 2013). In the present paper, we suggest that *H. influenzae* contains a PE-dependent storage pool of hemin, and this may result in a hemin supply to the *H. influenzae* population when there, for example, is a paucity of hemin in the environment.

Materials and methods

Bacterial strains and reagents

All NTHi strains (NTHi 3655 wild type, NTHi 3655Δ*hpe* and NTHi 3655*lux*) were routinely grown in brain heart infusion (BHI) liquid broth, supplemented with 10 µg/ml nicotinamide adenine dinucleotide (NAD) and 10 µg/ml hemin. NTHi were cultured with shaking at 200 rpm or on chocolate agar plates incubated at 37°C with 5% CO_2. The NTHi 3655Δ*hpe* was grown in the presence of 4 µg/ml zeomycine and the NTHi 3655*lux* in the presence of 10 µg/ml kanamycin. All strains were grown without antibiotics during co-culture. For hemin starvation, NTHi was grown in BHI medium supplemented with NAD but without hemin. *E. coli* BL21 (DE3) and DH5α were cultured in Luria–Bertani (LB) broth or on LB agar plates at 37°C in a humid atmosphere containing 5% CO_2. *E. coli* harboring pET26b*hpe* or pET16b*hpe* (Hallstrom et al., 2009; Singh et al., 2013) were grown in LB broth in the presence of 50 µg/ml kanamycin or 100 µg/ml ampicillin, respectively.

Construction of the luminescent NTHi (NTHi 3655lux) strain

The *lux* operon (*lux*ABCDE) from *Photorhabdus luminescens* subsp. laumondii TT01 was kindly provided by Dr. Nick Waterfield (University of Warwick, Coventry, UK) and stably inserted into the genome of NTHi 3655 following a strategy described previously (Fig. S1A) (Unal et al., 2012). The promoter of protein P5 was amplified by PCR using the primers promP5_F and promP5_R (Table S1) and cloned into pKR7.1 (Su et al., 2013) that yielded the plasmid pKR7.3 after digestion by restriction enzymes EcoRV and SalI. The *lux* gene was amplified from the genomic DNA of *P. luminescens* TT01 by using specific primers luxop_F and luxop_R (Table S1). The purified PCR product was inserted into the pKR7.3 vector between restriction sites

NheI and BglII to generate the vector pKR7.3*lux*ABCDE. At this stage the functionality of the *lux* operon was assessed by monitoring the chemiluminescence emitted by the *E. coli* clones with the Chemidoc XRS+ system (Biorad). Full *lux* operon along with the P5 promoter was amplified from pKR7.3*lux*ABCDE by using the KS36 and KS37 primer set, and the subsequent PCR product was used to transform the strain NTHi 3655 as described (Poje and Redfield, 2003). Transformants were selected on chocolate agar containing 10 μg/ml kanamycin. The presence of the operon in the resulting clones was verified by PCR (Fig. S1B and S1C).

Hemin-binding to the bacterial surface via PE

NTHi 3655 and NTHi 3655Δ*hpe* were collected from freshly prepared chocolate agar plates and inoculated in BHI medium supplemented with 10 μg/ml NAD only and grown for 8 h. Thereafter, bacteria were collected by centrifugation and resuspended in PBS ($OD_{600\ nm}$= 0.1). Subsequently, a fraction of this culture (100 μl) was added to 25 μl BHI medium supplemented with hemin and NAD followed by overnight incubation. Bacteria were collected, washed three times in PBS and photographed. For semi-quantitative determination of hemin present in NTHi 3655 and NTHi 3655Δ*hpe*, bacteria were spotted on PVDF membranes by using two-step serial dilutions. Hemin was detected by an enhanced chemiluminescence (ECL) western blot detection kit (Pierce). To define the hemin-binding capacity of PE, we expressed PE on the surface of *E. coli* BL21 using pET16*hpe* as described previously (Singh et al., 2011). *E. coli* expressing PE and control *E. coli* (transformed with the empty vector pET16b) were incubated in PBS with hemin at increasing concentrations (0 μM to 150 μM). After 1 h of incubation at room temperature, *E. coli* were washed

twice in PBS to remove the unbound hemin. Bacteria were finally resuspended into PBS and photographed. To assess the importance of available surface bound hemin for bacterial growth, NTHi 3655 and NTHi 3655Δ*hpe* were prepared exactly the same way as described above. After washing three times in PBS, the pellet was finally resuspended in BHI medium ($OD_{600\ nm}$= 0.1) containing 10 µg/ml NAD in the presence or absence of hemin. Cultures were incubated at 37 °C and shaking at 200 rpm, and bacterial growth was monitored at $OD_{600\ nm}$ every 30 min during 6 h.

Protein purification and preparation of the PE-hemin complex

Protein E was recombinantly expressed in *E. coli* and purified from the soluble fraction or inclusion bodies by using a HisTrap Ni^{++} NTA resin column (GE Healthcare Biosciences) as described previously (Singh et al., 2012; Singh et al., 2013). Hemin (Sigma) was prepared as a 5 mM stock solution in 1 M NaOH, and thereafter diluted 200-fold in Tris-HCl, pH 7.8 containing 100 mM NaCl (TS buffer) and stored at -20 °C. To analyze the hemin bound to PE, purified PE (25 µM) was incubated with hemin at increasing concentrations (0.1-2.5 mM) in a total volume of 20 µl in TS buffer. Samples were incubated for 1 h at RT and analyzed on a 6% native PAGE (Singh and Rohm, 2008). Hemin only (2.5 mM) was also loaded as a control. Gels were run at 80V using the gel electrophoresis Mini-PROTEAN Tetra System (Bio-Rad). PE and the PE-hemin complex were visualized by Coomassie R250 staining. For PE-hemin complex purification, first recombinant PE and hemin were mixed at a 1:2 molar ratio in TS buffer and incubated overnight at 4 °C to form the complex. The PE-hemin complex was loaded on a gel filtration column (Superdex 200) connected to an ÄKTAprime plus FPLC (GE Healthcare Biosciences) equilibrated with TS buffer, and was separated at a flow rate of 0.5 ml/min. Fractions

were collected and analyzed by native PAGE. The integrity of the PE-hemin complex in different fractions was also verified by a UV-visible spectrophotometer Ultrospec 2100 pro (GE Healthcare Bioscience). Fractions containing pure PE-hemin complex were pooled accordingly and concentrated by using 5 kDa MWCO Vivaspin® concentrator (Sartorius). Protein concentration in the PE-hemin complex solution was measured using the BCA kit (Pierce). Hemin bound to PE was quantified at 385 nm by using free hemin as a standard.

UV-visible spectrophotometry and PE-hemin binding analysis

Free hemin generates a characteristic Soret band at 385 nm that shifts towards 415 nm when hemin is bound to protein. We used this property of hemin to demonstrate the characteristics of hemin binding to PE using a UV-visible spectrophotometer (Vergauwen et al., 2010). Purified PE (15 μM) was incubated with 30 μM hemin in TS buffer for 1 h at room temperature and samples were scanned between 300 nm to 600 nm by using a UV-visible spectrophotometer. The background was corrected by 30 μM hemin (in TS buffer) in the reference cell. Figures were generated by using GraphPad Prism 6 software.

Surface plasmon resonance analysis

The affinity between PE and hemin was analyzed by surface plasmon resonance using a Biacore 2000 (GE Healthcare). Hemin was biotinylated prior to immobilization on a sensor chip. For biotinylation, 1.3 mg hemin was dissolved in 770 µl PBS, pH 7.5. Subsequently, 40 µl Biotin-PEG3 (100 mg/ml) and 190 µl of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (20 mg/ml) were added to the hemin solution. Hereby hemin was linked to biotin at its carboxy-side chains leaving the

central iron atom intact. The labeling reaction was performed for 3 h at room temperature with gentle shaking. Thereafter, the labeled hemin was dialyzed against PBS to remove unbound biotin and finally concentrated to 1 mg/ml by using 5 kDa MWCO Vivaspin® concentrator. The hemin and hemin-biotin binding to PE was verified by using native PAGE. Biotinylated hemin was immobilized on a SA (streptavidin) sensor chip (GE Healthcare). Biotinylated hemin (2 □g/ml) diluted in running buffer (50 mM HEPES, pH 7.8 containing 150 mM NaCl, 2 mM EDTA and 0.005 % Tween-20) was injected to achieve 500 resonance units (RU). Adjacent flow cell containing only streptavidin served as a negative control. The binding kinetics was studied for various concentrations of purified PE (0.19–6.0 μM). The signal from the control surface was subtracted. In all experiments, two consecutive injections of 2 M NaCl were used to remove bound ligands during a regeneration step. The BiaEvaluation 3.0 software (Biacore) was used to determine response at steady state for each sensorgram, which was thereafter plotted against PE the concentration. K_d was calculated using a steady state affinity equation.

Analysis of bacterial growth by using the PE-hemin complex as a source of hemin

NTHi 3655 were grown without hemin for 8 h in BHI medium supplemented with 10 μg/ml NAD only. Bacteria were washed three times in PBS and inoculated into fresh BHI ($OD_{600\ nm}$= 0.1) containing NAD (10 μg/ml). Free hemin or equimolar concentrations of PE-hemin complex were used as an iron source. Growth of bacteria was monitored every 30 min for 6 h by measuring absorbance ($OD_{600\ nm}$) using a cell density meter (Biowave CO8000, Biochrom). These cultures were also grown overnight (18 h) to ensure that most of the hemin was consumed in the culture medium, and to exclude dissociation of the PE-hemin complex. In addition, we

incubated the PE-hemin complex in BHI and NAD for 18 h in the absence of NTHi. To purify PE, or the PE-hemin complex, bacteria were spun down, and thereafter supernatants were loaded on a HisTrap Ni^{++}-resin column (GE Healthcare Biosciences). Purified samples were analyzed by a UV-visible spectrophotometer.

Co-culture of luminescent NTHi 3655lux with NTHi 3655 or NTHi 3655 Δhpe

NTHi 3655 or NTHi 3655 Δ*hpe* were co-cultured with NTHi 3655*lux* to analyze transfer of hemin between the strains. NTHi 3655 and NTHi 3655 Δ*hpe* were prepared as described above. In parallel, NTHi 3655*lux* was starved for hemin. For starvation, NTHi 3655*lux* was freshly grown on chocolate agar plates and inoculated in BHI medium containing only NAD (10 µg/ml) for 8 h at 37°C. The optical densities of all cultures were adjusted in BHI medium, and bacteria at equal numbers were mixed. Each reaction was performed in triplicates (final culture volume 1.3 ml) in a 96-well plate (Uniplate®, Whatman) followed by incubation at 37 °C. One sterile glass bead was added to each well for proper agitation during shaking at 200 rpm. The luminescence was monitored every hour in a 1450 MicroBeta TriLux counter (PerkinElmer).

Statistical analysis

Data were analyzed with the two-way analysis of variance (ANOVA) using the GraphPad Prism 6.0. A P value ≤ 0.05 was considered as statistically significant.

Results

Protein E binds hemin at the surface of Haemophilus influenzae and functions as a reservoir for hemin

It is a well-known fact that *H. influenzae* needs heme for growth and survival in the host (Infante-Rivard and Fernandez, 1993). Based upon the structural data of the PE molecule that appears as a dimer with a pocket (Singh et al., 2013), we screened several ligands including hemin that may possibly interact with PE. Molecular modeling indicated that the binding pocket may accommodate a hemin molecule (Fig. 1).

Since PE is a surface-exposed outer membrane protein (Ronander et al., 2009), we questioned if an *hpe* gene mutation would interfere with the hemin-binding capacity of *H. influenzae*. Importantly, we observed a difference in hemin-binding between wild type NTHi 3655 and the PE mutant NTHi 3566Δ*hpe* as visually verified in test tubes (Fig. 2A). Bacteria from cultures shown in Fig. 2A were also blotted on a nitrocellulose membrane and hemin was analyzed by using ECL (Fig. 2B). This semiquantitative determination indicated that more hemin had been acquired by NTHi 3655 in comparison to the PE mutant NTHi 3655Δ*hpe*. To further prove that PE binds hemin, recombinant PE was expressed at the surface of *E. coli.* In agreement with the results obtained with *H. influenzae* (Fig. 2A), addition of hemin to PE-expressing *E. coli*, led to more hemin acquisition in comparison to a control *E. coli* carrying an empty vector (Fig. 2C).

We also analyzed whether hemin bound to PE at the surface of NTHi would influence bacterial growth. The PE-expressing wild type NTHi and the corresponding PE mutant NTHi 3655Δ*hpe* were grown in medium supplemented with hemin overnight. After extensive washing steps to remove unbound hemin, the two NTHi

strains were separately incubated in culture medium without hemin. Bacterial growth was thereafter analyzed at the indicated time points (Fig. 2D). Interestingly, the NTHi 3655Δ*hpe* grew slower than NTHi 3655 in the absence of hemin, whereas growth of both strains was comparable in culture medium supplemented with hemin (10 μg/ml) (Fig. 2D). Taken together, our results suggest that PE plays a role as a hemin-binding outer membrane protein of *H. influenzae*, and may work as a storage pool for hemin.

Soluble recombinant H. influenzae PE binds hemin

To examine the direct hemin-binding capacity of PE, recombinant PE and hemin were mixed to form a complex followed by analysis by native PAGE. PE without hemin migrated slowly, whereas incubation of PE with increasing hemin concentrations resulted in formation of a PE-heme complex that migrated much faster through the native gel (Fig. 3A). UV-visible spectrophotometry also confirmed a direct interaction between PE and hemin (Fig. 3B). The absorption spectrum of hemin has a characteristic peak in the Soret band region that comprises a wavelength with visible blue light at ≈385 nm. A red shift of the Soret peak occurred with the PE-hemin complex, which was clearly observed at 415 nm (Fig. 3C). This suggested a PE-mediated perturbation of the electronic structure of the hemin iron.

The affinity of the hemin interaction with PE was analyzed by surface plasmon resonance (Biacore). Biotinylated hemin was immobilized on a sensor chip followed by injection of PE at increasing concentrations. A steady state affinity model suggested that hemin interacted with PE at $K_d \approx 1.6 \pm 0.27 \times 10^{-6}$ M (Fig. 3D). The surface protein F (PF) (Su et al., 2013) was included as a negative control and did not

bind to hemin (data not shown). Taken together, the *H. influenzae* PE has the capacity to attract hemin resulting in a stable complex formation.

H. influenzae captures hemin from a complex consisting of recombinant PE and hemin

To investigate whether hemin bound to recombinant PE can be used by *H. influenzae* and thereby promote bacterial growth, we incubated NTHi 3655 starved of hemin in the presence of the PE-hemin complex or equimolar free hemin. Interestingly, no difference in bacterial growth was observed between cultures supplemented with the PE-hemin complex or free hemin while in their absence the growth was completely inhibited (Fig. 4A).

To exclude that the PE-hemin complex dissociated during our culture conditions, the PE-hemin complex was incubated for up to 18 h at the same culture conditions but without NTHi. This time point was also chosen to ensure that NTHi had utilized all hemin from the PE-hemin complex. Culture samples were subjected to affinity chromatography using Ni^{++}-NTA resin columns. After elution and quantification, UV-visible spectrophotometry was performed. The integrity of the PE-hemin complex in the absence of NTHi was verified by a shift of the Soret peak towards the wavelength 415 nm (Fig. 4B). In contrast, no PE-hemin complex was detected in cultures with NTHi suggesting that hemin most likely had been consumed by the bacteria. The PE-hemin complex was thus stable and could be used as a hemin source for NTHi.

Hemin loaded on PE at the bacterial surface is donated to H. influenzae starved of hemin

Our experiments with NTHi 3655 and the PE mutant (Fig. 2D) suggested that PE may play a crucial role for the supply of hemin. To further demonstrate the usage of hemin derived from PE, we set up a co-culture with a luminescent NTHi (NTHi 3655*lux*) that was starved of hemin, and NTHi 3655 or NTHi 3655Δ*hpe* that both were cultured in the presence of hemin (Fig. 2A). Interestingly, NTHi 3655*lux* grew better when incubated with NTHi 3655 that carried more hemin at the surface as compared to the PE-deficient NTHi 3655Δ*hpe* (Fig. 5A). Growth of NTHi 3655*lux* in the presence or absence of supplemented hemin was included as a control in a parallel experiment (Fig. 5B). To summarize, our results indicate that hemin is shared between *H. influenzae* during shortage, that is, hemin-carrying NTHi may donate hemin to the co-cultured NTHi population.

Discussion

Iron is an essential element for almost all living organisms. As a catalytic center for redox reactions in many enzymes, iron facilitates numerous cellular processes such as electron transport, peroxide reduction, and nucleotide biosynthesis (Tong and Guo, 2009). As free iron is relatively insoluble and toxic, specific uptake pathways are present in almost all organisms. The iron uptake by Gram negative bacteria is energized by the TonB-ExbB-ExbD system, and iron is imported by ABC transporters. The TonB system of *H. influenzae* is involved in iron uptake mechanisms and is essential for virulence (Jarosik et al., 1995; Morton et al., 2012). Several pathogens also secrete high affinity iron-binding siderophores for iron retrieval from the surrounding tissue (Andrews et al., 2003). *H. influenzae* lacks the enzymes required in the biosynthetic pathway for the porphyrin ring, which is an immediate precursor of heme (Panek and O'Brian, 2002). *H. influenzae* expresses, however, ferrochelatase,

which mediates insertion of iron into protoporphyrin IX to produce heme (Loeb, 1995; Panek and O'Brian, 2002). Thus, *H. influenzae* utilizes hemin, hemoglobin, hemoglobin-haptoglobin, heme-hemopexin, transferrin and lactoferrin as iron sources. Since the species cannot grow in the absence of iron or porphyrin, both are indispensable for *H. influenzae* colonization (Gilder and Granick, 1947).

It is now apparent that *H. influenzae* has numerous mechanisms for binding and uptake of heme, irrespectively whether the heme is free or bound to a heme host carrier protein (Cope et al., 2000). However, in contrast to other bacterial species, *H. influenzae* does not produce any siderophores by itself, but can utilize siderophores produced by other microorganisms for iron uptake (Morton et al., 2010). The mechanisms for heme acquisition have not yet been fully elucidated for this bacterial species, but several heme-binding proteins of *Haemophilus* have been studied for their interaction with heme. Most of those are transporters or transport-associated proteins described as hemophores (Whitby et al., 2009). High heme content can be toxic for some pathogens, whereas several pathogens such as *Yersinia pestis*, *Aeromonas salmonicida*, *Shigella flexneri*, *Prevotella* spp., and *Porphyromonas* spp. are known for storing heme at their surface and utilize it for virulence (Anzaldi and Skaar, 2010). *H. influenzae* outer membrane lipoprotein P4 was previously described to play role in hemin uptake and transport (Reidl and Mekalanos, 1996). However, any hemin storage surface protein of *H. influenzae* has until now not been reported. Here we suggest that hemin is not only stored by PE at the bacterial surface but can also be utilized by other NTHi in the population. The hemin storage pool can thus also overcome the nutritional immunity and assist bacteria to survive in conditions related to heme paucity.

The equilibrium dissociation constant (K_d) of the PE-hemin binding affinity was $1.6x10^{-6}$ M as estimated by surface plasmon resonance, which one of the highest reported for any membrane protein of *H. influenzae*. Interestingly, the hemin-binding affinity for PE is higher than that of periplasmic heme-binding proteins such as HbpA ($K_d \approx 655x10^{-6}$ M) and SapA ($K_d \approx 56x10^{-6}$ M) (Jin et al., 1996; Jin et al., 1999). However, FbpA binds ferric iron with an affinity that was estimated to $K_d = 2.0x10^{-18}$ M, and FbpA furthermore extracts iron from the human high affinity iron-binding protein transferrin (Khan et al., 2007). Thus, based upon the binding affinity and comparison with other iron acquiring proteins of *H. influenzae*, that is, HbpA, SapA and FbpA, PE has an optimal affinity for hemin to function as a hemin carrier for *H. influenzae*. We hypothesize that PE may attract hemin from the surroundings in the bacterial niche when there is excess hemin available during, for example, tissue destruction linked to infection, smoking-induced damage or inflammation in COPD patients.

In conclusion, this report reveals a unique function attributed to the multifunctional *H. influenzae* PE. The involvement of PE in hemin acquisition was proven by analysis of a NTHi mutant devoid of PE in addition to surface expression of the heterologous host *E. coli*. This is to our knowledge the first experimental evidence on distribution of hemin between *H. influenzae* and sheds light upon the mechanisms that are related to nutrient sharing.

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Legends

Fig. 1. Putative docking of a hemin molecule in the binding pocket of the PE-dimer. Surface representation of PE crystal structure (Singh et al., 2012; Singh et al., 2013) with a hypothetical model of the PE-hemin complex. Insert: zoom in projection of a putative hemin-binding pocket in dimeric PE. Docking was performed manually using COOT (Emsley et al., 2010; McNicholas et al., 2011; Potterton et al., 2002). The figures were prepared with Pymol and CCP4MG.

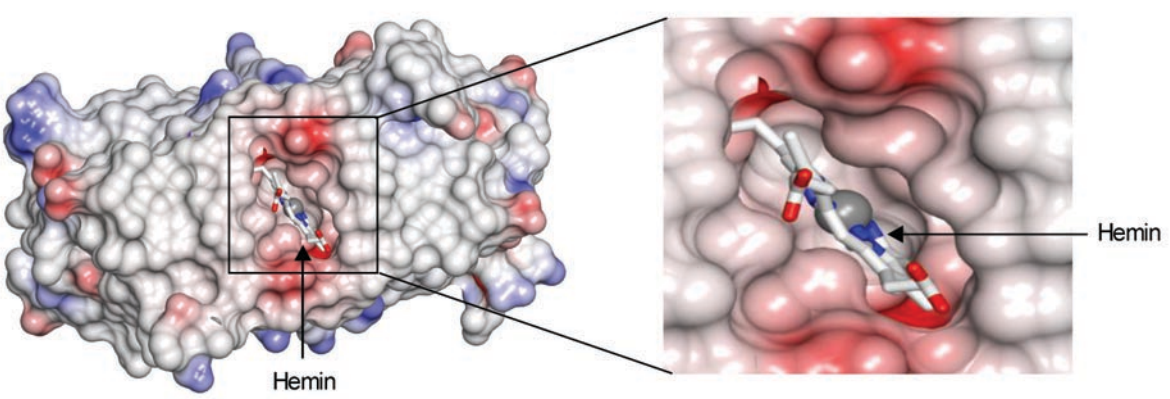
Fig. 2. *Haemophilus influenzae* PE binds hemin at the bacterial surface and utilizes it when needed. (A) Hemin binding to NTHi 3655 and NTHi 3655Δ*hpe*. Bacteria were grown overnight in BHI with hemin and NAD (both at 10 μg/ml). After washing three times in PBS, 5 ml of each culture ($OD_{600\ nm}$=1 corresponding to 10^9 NTHi) of each strain were resuspended in 1 ml PBS. (B) Semi quantitative measurement of PE-dependent hemin-binding on the surface of wild type NTHi 3655 as compared to the PE mutant NTHi 3655Δ*hpe*. Bacteria shown in panel A were serially diluted and spotted on a PVDF filter and ECL was used for detection of hemin on blots. (C) *E. coli* expressing PE at the surface bound hemin. *E. coli* expressing PE and control *E. coli* with an empty vector were incubated at room temperature with increasing concentrations of hemin. After 1 h incubation, bacteria were washed, resuspended in PBS and photographed. (D) Growth of NTHi 3655 and NTHi Δ*hpe* in BHI with NAD in the presence or absence of hemin. Before initiation of the culture for determining a growth curve, NTHi strains were incubated overnight with hemin and NAD followed by three washes in PBS. All experiments were repeated three times. The data in D represent mean values of three independent experiments with error bars indicating standard deviations (SD).

Fig. 3. Protein E of *H. influenzae* is a hemin-binding protein. (A) Native-PAGE gel illustrating binding of hemin to PE. PE (25 μM) was pre-incubated with hemin (0-2.5 mM) and subjected to a native PAGE followed by staining with Coomassie blue R250. The PE-hemin complex migrated faster than only PE. (B) The UV-visible spectra demonstrate the deviation in absorbance spectrum of 30 μM hemin solution when mixed with PE. PE (15 μM) incubated with hemin (30 μM) showed a shift of the peak towards 415 nm. (C) UV-visible spectrum of the PE-hemin complex. Complexes of 10 μM PE with 30 μM hemin was scanned and analysed. Hemin (30 μM) was used in the reference cell and values presented were adjusted with respect to this background measurement. This spectrum showed that the Soret band for unbound hemin was at 385 nm and shifted to 415 nm upon interaction with PE. (D) Surface plasmon resonance analysis of PE and hemin. Biotinylated hemin was immobilized on a SA sensor chip and PE at increasing concentrations was injected. Sensograms for all PE concentrations (PE at 0.19–6.0 μM) are shown in the inset. The measurements were performed using a Biacore 2000. The response units at saturation were plotted against PE concentrations and fitted using a steady state affinity model and the Biaevaluation software.

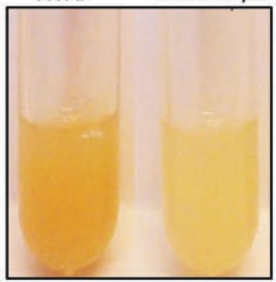
Fig. 4. Hemin is acquired by *H. influenzae* from the PE-hemin complex in solution. (A) Growth of NTHi 3655 in culture medium supplemented with free hemin, or equimolar concentrations (7.6 μM corresponding to 5 μg/ml) of the PE-hemin complex as compared to NTHi incubated in the absence of hemin. NAD was included in all cultures. (B) UV-visible spectrophotometry of samples from the experiment outlined in (A). Hemin was bound to PE as an integrated PE-hemin complex in

control cultures that were incubated without NTHi for 18 h. In contrast, hemin was extracted from the PE-hemin complex when incubated for 18 h with NTHi resulting in free PE in the supernatants. This experiment was repeated three times, and one representative experiment is shown here.

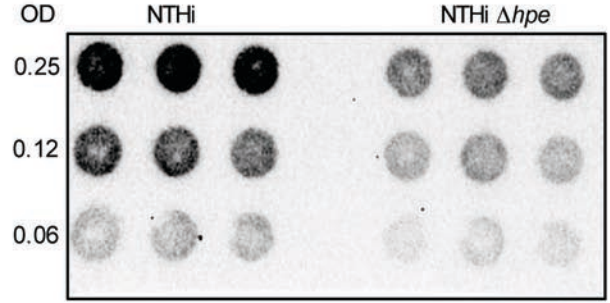
Fig. 5. Hemin bound to PE at the surface of *H. influenzae* is shared with co-cultured NTHi. (A) Growth of NTHi 3655*lux* was significantly promoted by NTHi 3655 as compared to NTHi 3655Δ*hpe* that was devoid of PE. To provide hemin, 10^8 cfu of NTHi 3655 or PE-mutant NTHi 3655Δ*hpe* were added to 10^8 luciferase-producing NTHi 3655*lux*. (B) Growth of NTHi 3655*lux* with or without supplemented hemin. NTHi 3655*lux* was grown in BHI medium containing 10 μg/ml each of hemin and NAD. In (A), NTHi 3655 and the PE-mutant NTHi 3655Δ*hpe* were first grown in BHI supplemented with hemin and NAD (both at 10 μg/ml). After three washings in PBS, the NTHi 3655*lux* was co-cultured with either NTHi or NTHi 3655Δ*hpe* at a ratio of 1:1. Results are shown as luminescence related to growth of NTHi 3655*lux*. The data represent mean values of three independent experiments in triplicates. Error bars indicate SD. Statistical significance of differences was calculated using a two-way ANOVA; $^{\star\star\star}P \leq 0.001$.


Hemin
Hemin

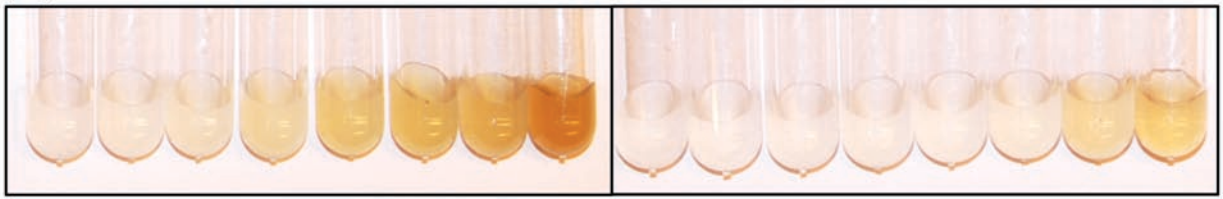
A


NTHi
NTHi Δ*hpe*

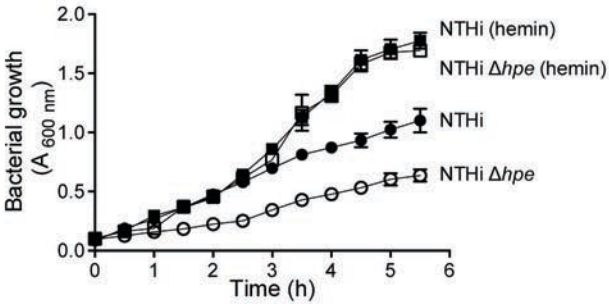
B

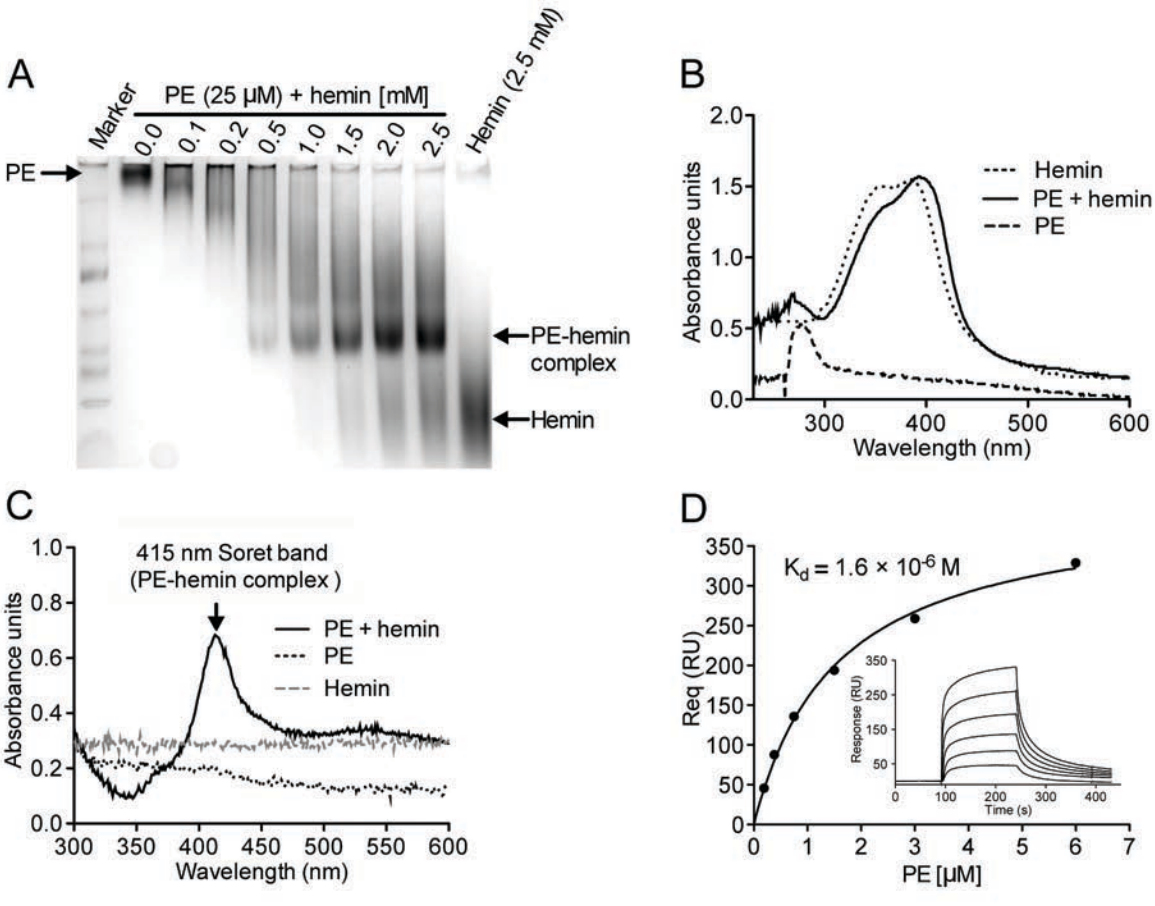

OD
NTHi
NTHi Δ*hpe*
0.25
0.12
0.06

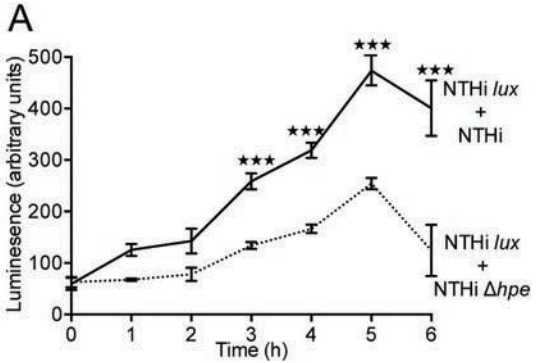
C

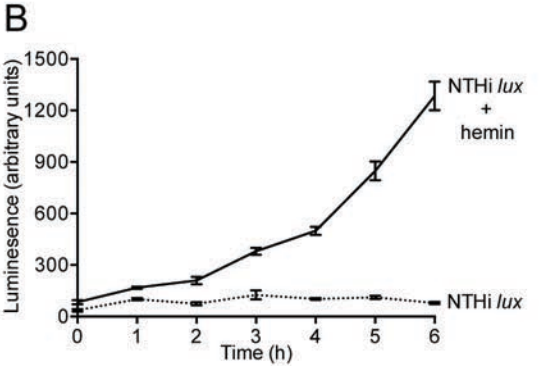

E. coli expressing PE
E. coli control
Hemin [μM] 0 5 10 20 50 75 100 150
0 5 10 20 50 75 100 150

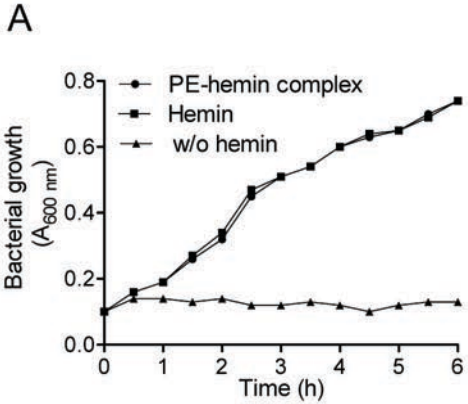
D

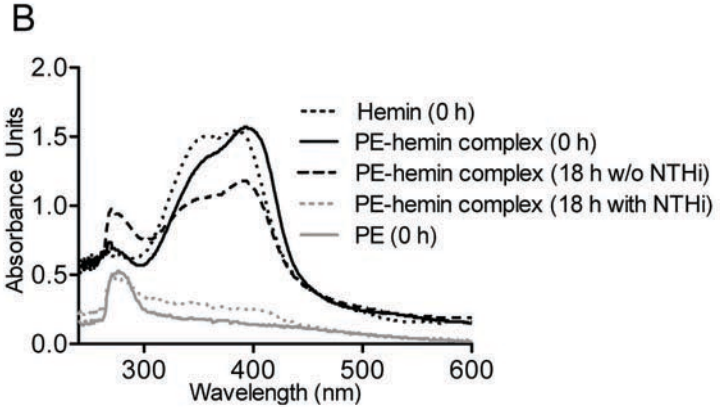

Bacterial growth ($A_{600\ nm}$)
2.0
1.5
1.0
0.5
0.0
0 1 2 3 4 5 6
Time (h)
NTHi (hemin)
NTHi Δ*hpe* (hemin)
NTHi
NTHi Δ*hpe*


A
Marker
PE (25 μM) + hemin [mM]
0.0
0.1
0.2
0.5
1.0
1.5
2.0
2.5
Hemin (2.5 mM)
PE
PE-hemin complex
Hemin
B
Absorbance units
Wavelength (nm)
Hemin
PE + hemin
PE
C
415 nm Soret band
(PE-hemin complex)
PE + hemin
PE
Hemin
Absorbance units
Wavelength (nm)
D
$K_d = 1.6 \times 10^{-6}$ M
Req (RU)
PE [μM]
Response (RU)
Time (s)


A
Luminesence (arbitrary units)
500
400
300
200
100
0
0
1
2
3
4
5
6
Time (h)
***
***
***
***
NTHi lux
+
NTHi
NTHi lux
+
NTHi Δhpe


B
Luminesence (arbitrary units)
1500
1200
900
600
300
0
0
1
2
3
4
5
6
Time (h)
NTHi lux
+
hemin
NTHi lux


A
PE-hemin complex
Hemin
w/o hemin
Bacterial growth
($A_{600\ nm}$)
0.0
0.2
0.4
0.6
0.8
0
1
2
3
4
5
6
Time (h)


B
Hemin (0 h)
PE-hemin complex (0 h)
PE-hemin complex (18 h w/o NTHi)
PE-hemin complex (18 h with NTHi)
PE (0 h)
Absorbance Units
0.0
0.5
1.0
1.5
2.0
300
400
500
600
Wavelength (nm)

Appendix A. Supplementary data

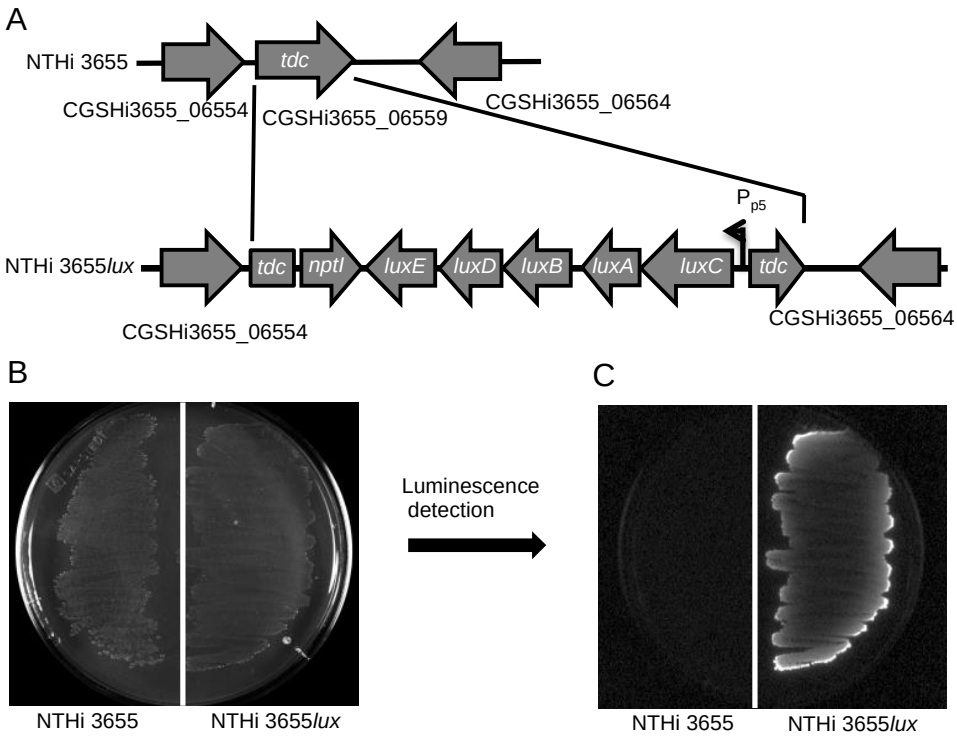


Fig. S1. Insertion of the *P. luminescens lux* operon in the genome of NTHi 3655. (A) Diagram depicting the strategy employed to insert the lux operon (*lux*ABCDE) from luminescens TT01 into NTHi 3655 genome. Genomic insertion was done in the locus CGSHi3655_06559 annotated as threonine dehydratase (*tdc*) in the draft genome of NTHi 3655. (B) NTHi 3655 and NTHi 3655*lux* luminescent clones grown overnight on GCG plates. (C) Luminescence was observed on a chemidoc XRS+ system (Biorad).

Table S1. Primers used for construction of the NTHi 3655*lux* strain

Primer	Sequence (5'-3')	Restriction enzyme
PromP5_F	CGCGATATCTGAAGGTAAAACGGCAGG	EcoRV
PromP5_R	TAACGTCGACAGATCTGGCGCGCCTAGGCTAGCTTTAGTCATCGAATAGTAATAAA	SalI
Luxop_F	AATCTATCGCTAGCTAAAT**AAGGA**CATCTTATGACTAAAAAAATTTCATTCATT	NheI
Luxop_R	CTCTGTCGACAGATCTGGAATCAACTATTAAATGCTT	BglII
KS36	GGCGGATCCTG**ACCGCACTT**AGGGGGATAAAACAAAGG	
KS37	GGCCTCGAG**AAGTGCGGT**CAGGCAAGTCCCTGTTCAAA	

The restriction enzyme sites used for cloning of the inserts are underlined. The ribosome binding site (in Luxop_F) and uptake signal sequences (in KS36 and KS37) are highlighted in bold.