Identification of a haem-utilization protein (Hup) in *Haemophilus influenzae*

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Haemophilus influenzae has an absolute growth requirement for a porphyrin source. This growth requirement can be satisfied *in vitro* by haem, haemoglobin or the haemoglobin–haptoglobin, haem–haemopexin and haem–albumin complexes. A family of proteins, termed the Hgp proteins, which are essential for utilization of the haemoglobin–haptoglobin complex, has previously been identified. A strain lacking the Hgp proteins also has a residual ability to utilize haemoglobin, indicating that additional moieties contribute to haemoglobin utilization. Using a haemoglobin affinity method an approximately 105 kDa protein was isolated. Mutation of the identified gene in an Hgp null background reduced the ability of the mutant strain to utilize haemoglobin *in vitro*. The mutation also resulted in a reduced ability to utilize haem, haem–haemopexin, haem–albumin and haemoglobin–haptoglobin, thus identifying a general haem-utilization protein (Hup) in *Haemophilus influenzae*.

INTRODUCTION

Haemophilus influenzae are fastidious Gram-negative bacteria that cause a range of human infections including otitis media, meningitis, epiglottitis and pneumonia (Foxwell *et al.*, 1998; Sethi & Murphy, 2001; St Geme, 2001; Turk, 1984). The incidence of meningitis caused by *H. influenzae* strains with the type b capsule has decreased by more than 90 % in industrialized countries following the introduction of vaccines based on the type b capsule (Hviid & Melbye, 2004; Watt *et al.*, 2003). *H. influenzae* strains of other capsular serotypes infrequently cause invasive disease, although recent reports indicate an increased incidence of invasive disease caused by *H. influenzae* of capsular serotypes other than b subsequent to the introduction of the type b vaccine (Bajanca *et al.*, 2004; Ribeiro *et al.*, 2003). Strains of *H. influenzae* lacking a capsule (nontypable *H. influenzae*) rarely cause invasive disease but are significant causes of otitis media in children and pneumonia in patients with predisposing conditions (Murphy, 2003; St Geme, 2001; Turk, 1984). Understanding the pathogenic

mechanisms of *H. influenzae* disease is important as a basis for treatment and eradication of this pathogen.

Since *H. influenzae* lacks all the enzymes in the biosynthetic pathway for the porphyrin ring, it is unable to synthesize protoporphyrin IX (PPIX), the immediate precursor of haem (Panek & O'Brian, 2002; White & Granick, 1963). However, most *H. influenzae* strains possess a gene encoding the enzyme ferrochelatase that mediates insertion of iron into PPIX to form haem (Loeb, 1995; Schlor *et al.*, 2000). Thus, *H. influenzae* has an absolute growth requirement for an exogenous PPIX or haem source (haematin is correctly ferrous PPIX while haemin is ferric PPIX; however, in this paper the term haem is used regardless of iron valency).

Since the only known niche for *H. influenzae* is humans, the organism must have adapted its mechanisms of haem acquisition accordingly. There is no significant source of free PPIX available in the normal human host. Haem is generally intracellular, in the form of haemoglobin or haem-containing enzymes, and therefore unavailable to invading micro-organisms (Genco & Dixon, 2001; Griffiths, 1999). Free haemoglobin, derived from lysed erythrocytes, is bound by the serum protein haptoglobin, and the haemoglobin–haptoglobin complex is rapidly cleared by the reticulo-endothelial cells of the liver, bone marrow or spleen (Evans *et al.*, 1999; Ward & Bullen, 1999). Free haem, principally derived from the degradation of methaemoglobin, is bound

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Abbreviation: PPIX, protoporphyrin IX.

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by the serum proteins haemopexin and albumin and cleared from the circulation (Genco & Dixon, 2001; Peters, 1996). Haemoglobin and the haemoglobin-haptoglobin, haem-haemopexin and haem-albumin complexes, as well as PPIX in the presence of an iron source such as ferritransferrin, can each be utilized by *H. influenzae* as haem sources *in vitro* (Morton & Williams, 1989; Schryvers & Gray-Owen, 1992; Stull, 1987).

A complex array of haem-uptake mechanisms has evolved in *H. influenzae* to ensure acquisition of this essential nutrient (Morton & Stull, 2004). For example, the *hxcBA* gene cluster mediates utilization of low levels of free haem, the haem-haemopexin complex and haemoglobin (Cope *et al.*, 1995, 1998, 2001), as well as the haem-albumin complex (D. J. Morton, L. L. Madore, T. M. VanWagoner, T. W. Seale, P. W. Whitby & T. L. Stull, unpublished observations), and utilization of ferritransferrin is mediated by the transferrin-binding proteins, Tbp1 and Tbp2 (Gray-Owen & Schryvers, 1996). We have shown that utilization of haemoglobin-haptoglobin is mediated by a family of haemoglobin/haemoglobin-haptoglobin-binding proteins (*hgpA*, *hgpB* and *hgpC* in the type b strain HI689) (Jin *et al.*, 1996, 1999; Morton *et al.*, 1999; Ren *et al.*, 1998).

Individual *H. influenzae* strains possess one to four *hgp* genes (Cope *et al.*, 2000; Morton *et al.*, 1999; Morton & Stull, 1999). Expression of the proteins encoded by the *hgp* genes is phase variable due to a length of CCAA nucleotide repeats immediately following the sequence encoding the leader peptide. Alterations in the length of the CCAA nucleotide repeat regions lead to frame-shift mutations and the introduction of stop codons (Ren *et al.*, 1999). While expression of the Hgp proteins is essential for the utilization of the haemoglobin-haptoglobin complex *in vitro*, mutants lacking the *hgp* genes retain the ability to utilize haemoglobin albeit at a reduced level (Morton *et al.*, 1999). These data demonstrate that although the Hgp proteins play a role in the utilization of haemoglobin, additional moieties are involved in the utilization of this haem source.

The objective of this study was to identify additional protein(s) involved in the utilization of haemoglobin and/or other potential haem sources by *H. influenzae*. We report the identification of an *H. influenzae* protein involved in the utilization of multiple haem sources that we have designated a haem-utilization protein (Hup).

METHODS

Bacterial strains, growth conditions and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. *H. influenzae* type b strain HI689 is a clinical blood isolate and has been previously described (Jin *et al.*, 1996; Musser *et al.*, 1986). Strain HI1717 is a derivative of strain HI689 lacking the *hgp* genes (*hgpA*, *hgpB*, *hgpC*) and was the subject of a previous report (Morton *et al.*, 1999). *H. influenzae* was routinely maintained on chocolate agar with bacitracin (BBL) at 37 °C. When necessary, *H. influenzae* was grown on brain heart infusion (BHI) agar (Difco) supplemented with 10 µg haem ml⁻¹ and 10 µg β-NAD

ml⁻¹ (supplemented BHI; sBHI) and the appropriate antibiotic(s). Haem-deplete growth was performed in BHI broth supplemented only with 10 µg β-NAD ml⁻¹ (haem-deplete BHI; hdBHI). *H. influenzae* was transformed to antibiotic resistance using a modification of the static aerobic method of Gromkova *et al.* (1989) as previously described (Morton *et al.*, 2004).

Haem sources. Human haemoglobin, human haptoglobin (from pooled sera), human serum albumin (HSA) and bovine haemin were purchased from Sigma. Stock haem solutions were prepared at 1 mg haem ml⁻¹ in 0.02 M NaOH. Haemoglobin was dissolved in water immediately before use. Haemoglobin-haptoglobin complexes were prepared as previously described (Morton *et al.*, 1999). Haem-albumin complexes were made by mixing 100 µg haem and 20 mg HSA per ml of water as previously described (Stull, 1987).

Rabbit haemopexin was prepared as described previously and the haem-haemopexin complexes were characterized by the typical features of their absorption spectra, which include the prominent shoulder at 290 nm that appears upon haem binding (Smith, 1985; Smith & Morgan, 1984).

DNA methodology. Restriction endonucleases were obtained from New England Biolabs and used as directed by the manufacturer. Genomic DNA was isolated using the DNeasy Tissue Kit (Qiagen) as directed by the manufacturer. Plasmid DNA was isolated using Wizard Plus Minipreps DNA purification system (Promega) according to the manufacturer's directions. Sequencing of double-stranded template DNA was performed by automated sequencing on an ABI Prism model 3700 DNA Analyser at the Recombinant DNA/Protein Resource Facility, Oklahoma State University, Stillwater, OK, USA. Oligonucleotides were synthesized by Qiagen.

Affinity isolation of haemoglobin-binding proteins. Outer-membrane proteins were purified and subjected to haemoglobin affinity purification as previously described (Jin *et al.*, 1996). Eluted proteins were separated by SDS-PAGE on 7.5% acrylamide gels using the discontinuous buffer system of Laemmli (1970). Approximately 30 µl of protein preparation, representing proteins isolated from approximately 10⁶ organisms, was loaded per lane.

N-terminal amino acid sequencing. Affinity-chromatography-purified proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane as previously described (Jin *et al.*, 1996). The entire membrane was submitted to the UCLA Medical School Protein Microsequencing Facility for N-terminal amino acid sequencing.

Cloning and sequencing of *hup* from *H. influenzae* type b strain HI689. Two separate primer pairs (Table 2) were designed for use in the PCR based on the Rd KW20 genomic sequence (Fleischmann *et al.*, 1995). These primer pairs amplified two overlapping fragments encompassing the ORF HI1217 locus. 1217A and 1217REVSQ2 amplified an ~1000 bp product encompassing approximately 800 bp encoding the C-terminal portion of the encoded protein and 200 bp downstream of the gene. Primers 1217SQ2 and 1217SQ101 amplified a product of ~2800 bp encompassing approximately 2000 bp encoding the N-terminal region of the encoded protein and approximately 800 bp upstream of the gene.

PCRs were performed in a 50 µl volume using 100 ng of *H. influenzae* HI689 chromosomal DNA as template, and the reactions contained 2 mM MgCl₂, 200 µM of each deoxynucleoside triphosphate (New England Biolabs), 10 pmol of each primer and 2 U of Taq DNA Polymerase (Roche). PCR was carried out for 30 cycles, with each cycle consisting of denaturation at 95 °C for 1 min, annealing for 1 min at 58 °C for primer pair 1217A/1217REVSQ2 and 57 °C for primer pair 1217SQ2/1217SQ101, and primer extension at 72 °C for 1 min, with

Table 1. Strains and plasmids

Strain or plasmid	Relevant characteristic(s)*	Reference or source
Strains		
<i>H. influenzae</i>		
HI689	Type b	Musser <i>et al.</i> (1986)
HI1714	HI689, $\Delta hgpA \Delta hgpB$, Rb ^r Tc ^r	Morton <i>et al.</i> (1999)
HI1717	HI689, $\Delta hgpA \Delta hgpB \Delta hgpC$, Rb ^r Tc ^r Sp ^r	Morton <i>et al.</i> (1999)
HI1737	HI689 Δhup , Zeo ^r	This study
HI1738	HI1717 Δhup , Zeo ^r	This study
HI1955	HI1737, pDJM367, Zeo ^r Cm ^r	This study
HI1953	HI1738, pDJM367, Rb ^r Tc ^r Sp ^r Zeo ^r Cm ^r	This study
El1a	Type b	Stull <i>et al.</i> (1984)
<i>E. coli</i>		
TOP10	Used for cloning in pCR2.1-TOPO and pUC19N	Invitrogen
Plasmids		
pCR2.1-TOPO	Plac, lacZx, Kan ^r Amp ^r , ColE1 origin, F1 origin, T7 promoter	Invitrogen
pUC19N	pUC19 with a NotI site added at the HindIII end of the polylinker	Tartof & Hobbs (1988)
pSU2718	<i>E. coli</i> - <i>H. influenzae</i> shuttle vector, lacZx, Cm ^r , p15a ori	Martinez <i>et al.</i> (1988)
pEM7/Zeo	Plasmid expressing Zeo ^r (<i>Sh ble</i>) using the bacterial EM7 promoter; the Zeo ^r cassette is flanked by polylinkers to allow excision of the resistance marker	Invitrogen
p1217UD	pUC19N carrying an ~1100 bp PCR product corresponding to a region upstream of <i>hup</i> and an ~1100 bp product corresponding to a region downstream of <i>hup</i> with a unique BamHI site between the two inserts	This study
p1217UD-ZEO	p1217UD with the ~700 bp Zeo ^r marker from pEM7/Zeo inserted at the unique BamHI site	This study
pDJM359	pCR2.1-TOPO carrying an ~3200 bp PCR product encompassing the <i>hup</i> coding sequence and upstream and downstream regions	This study
pDJM367	pSU2718 carrying the 3200 bp PCR product from pDJM359	This study

*Amp^r, ampicillin resistance (50 µg ampicillin ml⁻¹ in *E. coli*); Cm^r, chloramphenicol resistance (1.5 µg chloramphenicol ml⁻¹ in *H. influenzae* and 50 µg chloramphenicol ml⁻¹ in *E. coli*); Kan^r, kanamycin resistance (50 µg kanamycin ml⁻¹ in *E. coli*); Sp^r, spectinomycin resistance (200 µg spectinomycin ml⁻¹ in *H. influenzae*); Tc^r, tetracycline resistance (3 µg tetracycline ml⁻¹ in *H. influenzae*); Zeo^r, zeocin resistance (50 µg zeocin ml⁻¹ in *H. influenzae* and 100 µg zeocin ml⁻¹ in *E. coli*).

one final extension of 30 min. PCR products of the expected size were obtained from both PCRs and were successfully cloned into the TA cloning vector pCR2.1-TOPO. The insert DNA sequence derived from at least two independent PCRs was determined by automated sequencing. Discrepancies were resolved by the sequencing of a third independent PCR product across the appropriate region.

Table 2. Primers

Primer	Sequence (5'–3')
1217REVSQ2	CTCAAGACGATGTGCTAGG
1217A	GTTCGAGCAATTTCTGATGG
1217SQ2	TAGCACATCGTCTTGAGTG
1217SQ101	CTTCTTTACGTACCCGC
1217-1	GGATCCTTATCCATTTTATTAAGAGG
1217-2	CTGCAGGCTTATTTATTTCTGTATGGG
1217-3	GGATCCGCATAGCTAATAGCTATGCC
1217-4	GAATTCGGATAACCAAGTTTAGCG
1217-5	GAATTCCTTAGTACCTTTATTTTATAATC
1217-6	GGATCCCTTAGTACCTTTATTTTATA

Construction of an *hup* deletion mutant. Complete deletion of *hup* in strains HI689 and HI1717 was achieved essentially as previously described for deletion of the *hgp* genes (Morton *et al.*, 1999). Four primers (Table 2) for use in the PCR were designed based on the available *H. influenzae* strain Rd KW20 genomic sequence (Fleischmann *et al.*, 1995) to delete *hup* in strain HI689. Primers 1217-1 and 1217-2 were designed to amplify a product of approximately 1100 bp upstream of *hup* and, respectively, add BamHI and PstI sites to the ends of the amplicon to allow for subsequent directional subcloning. Primers 1217-3 and 1217-4 were designed to amplify an approximately 1100 bp product downstream of *hup* and, respectively, add BamHI and EcoRI to the ends of the amplicon to allow for directional subcloning.

PCR was carried out as described above but with annealing for 1 min at 53 °C for primer pair 1217-1/1217-2 and at 47 °C for primer pair 1217-3/1217-4. PCR products of the expected size were obtained from both PCRs and were successfully cloned into the TA cloning vector pCR2.1-TOPO. The cloned amplicon from each reaction was confirmed as correct by automated DNA sequencing. Utilizing the engineered restriction sites, the PCR products were successively subcloned into pUC19N to yield p1217UD. Plasmid p1217UD thus comprised upstream and downstream sequences of HI1217 abutting each other, with a unique BamHI site engineered between the upstream and downstream regions. The zeocin-resistance marker from pEM7/Zeo

was excised by digestion with *Bam*HI and *Bgl*II and cloned into the unique *Bam*HI site of p1217UD to yield p1217UD-ZEO. Competent *H. influenzae* was transformed with p1217UD-ZEO and selected on chocolate agar with zeocin spread on the surface of the plate. The initial selection of recombinant *H. influenzae* was at 25 µg zeocin ml⁻¹, with subsequent subculture of potential transformants at 50 µg zeocin ml⁻¹. Correct chromosomal rearrangements were confirmed by the molecular size of a PCR product resolved on an agarose gel (data not shown).

Complementation of mutant strains. To complement the deletion of *hup* in the mutant strains a plasmid was constructed carrying the entire *hup* gene. A 3200 bp PCR product, encompassing the entire *hup* coding sequence and 280 bp upstream of the start codon and 150 bp downstream of the stop codon, was amplified from HI689 chromosomal DNA using primers 1217-5 and 1217-6 (annealing at 50 °C for 1 min, extension for 2 min). An amplicon of the expected size was cloned into pCR2.1-TOPO to yield pDJM359 and confirmed by automated DNA sequencing. pDJM359 was digested with *Kpn*I and *Sph*I and the band corresponding to the chromosomally derived insert was ligated to *Kpn*I/*Sph*I-digested pSU2718, a shuttle vector with the p15a origin of replication that allows establishment of the plasmid in *H. influenzae*, to yield pDJM367. pDJM367 was confirmed by automated DNA sequencing, and was electroporated into *H. influenzae* *hup* deletion mutant strains to yield the corresponding merodiploid strains. Electroporation of *H. influenzae* was carried out as previously described (VanWagoner *et al.*, 2004) and transformants were selected on 1.5 µg chloramphenicol ml⁻¹.

Growth studies with *H. influenzae*. *H. influenzae* was grown for 12–14 h on chocolate agar with bacitracin, and these 12–14 h cultures were used to inoculate 10 ml hdBHI cultures that were incubated for 4 h at 37 °C with shaking (175 r.p.m.). The 4 h cultures were pelleted by centrifugation, washed once in 0.1% (w/v) gelatin in PBS and resuspended to an optical density at 605 nm of 0.5 in 0.1% (w/v) gelatin in PBS. One millilitre of the bacterial suspension was diluted in 5 ml of 0.1% (w/v) gelatin in PBS, and this final bacterial suspension was used to inoculate fresh hdBHI (0.1%, v/v, inoculum to give an approximate initial concentration of 200 000 c.f.u. ml⁻¹) supplemented as appropriate. Viable counts were performed on all cultures at time zero to ensure that all inocula were comparable (statistical comparisons were made using Student's *t*-test; no inocula were statistically different). Cultures were incubated at 37 °C with shaking (175 r.p.m.). Growth was monitored to stationary phase by measurement of the OD₆₀₅ with a Shimadzu UV-1201S spectrophotometer. Growth studies were performed in triplicate a minimum of twice for each condition. All growth studies included a negative control in hdBHI with no added haem source; no strain exhibited any increase in OD₆₀₅ in this negative control test.

Animals. Specific-pathogen-free (SPF) timed-pregnant Sprague-Dawley rats (Harlan Sprague-Dawley) were received approximately 5 days prior to giving birth. These pregnant females were single-housed on hardwood litter with *ad libitum* access to water and a standard pelleted food (Purina Lab Rodent Diet 5001). They were maintained on a 12 h light–dark cycle in separate forced-air cubicles in a bio-containment facility to prevent cross-contamination. Newborn pups from different mothers were pooled and randomly reassigned to the mothers (*n* = 10 pups per female). The protocol for usage of animals in this study was reviewed and approved by the Institutional Animal Use and Care Committee of the University of Oklahoma Health Sciences Center.

Infection of animals, collection of blood samples and quantification of bacteraemia. The infant rat model for haematogenous meningitis following intraperitoneal infection with *H. influenzae*

(Smith *et al.*, 1973) was used to compare the abilities of strains to cause bacteraemia. The inoculum was prepared as follows. *H. influenzae* was incubated for 18 h at 37 °C on chocolate agar containing bacitracin. A loopful of these cells was used to inoculate 5 ml hdBHI. These broth cultures were incubated at 37 °C with gentle rotation for approximately 4 h until moderately turbid. Bacteria were pelleted by centrifugation, washed once with PBS containing 0.1% (w/v) gelatin, and the pelleted cells were resuspended in the same buffer. The suspension was adjusted to an OD₆₀₅ of 0.5 and then diluted serially in the same solution to provide the standard inoculum (200 c.f.u. in 100 µl) that was injected intraperitoneally into 5-day-old rat pups. To determine the actual infective dosage, 100 µl aliquots of the inoculum were plated on chocolate agar containing bacitracin.

At 24 h intervals pups were examined for symptoms of infection, and blood specimens (50 µl) were obtained. Bacteraemia was quantified using a modification of the track-dilution procedure of Jett *et al.* (1997). Serial dilutions (0 to 10⁻⁵) of freshly drawn whole-blood specimens were made with PBS containing 0.1% (w/v) gelatin. Aliquots of 10 µl from each dilution were plated in triplicate on chocolate agar plus bacitracin and incubated at 37 °C for 24 h prior to counting.

Statistics. Statistical comparisons of growth between strains under the same growth conditions were assessed using the Kruskal–Wallis test. In some cases the analysis was performed over the entire growth curve and in others only over the period of active growth as specified in the text. Analyses were performed using Analyse-It for Microsoft Excel v1.71 (Analyse-It Software).

For *in vivo* studies bacteraemic titres are expressed as means ± SD typically from groups of 10 animals. Statistical comparisons of mean bacteraemic titres between two groups of animals infected with different strains were assessed with the unpaired Student's *t*-test. Analyses were performed with SigmaStat software (SPSS). A *P* value < 0.05 was taken as statistically significant in all analyses.

RESULTS AND DISCUSSION

Isolation and N-terminal amino acid sequencing of haemoglobin-binding proteins

We previously identified a family of haemoglobin/haemoglobin–haptoglobin-binding proteins in *H. influenzae* (Jin *et al.*, 1999; Morton *et al.*, 1999; Ren *et al.*, 1998). Different strains of *H. influenzae* possess different complements of these haemoglobin/haemoglobin–haptoglobin-binding proteins (HgpA, HgpB and HgpC in *H. influenzae* type b strain HI689) (Cope *et al.*, 2000; Morton *et al.*, 1999). Complete deletion of the Hgp complement of *H. influenzae* strains renders the mutant strain unable to utilize haemoglobin–haptoglobin complexes as a haem source, though still able to utilize haemoglobin as a haem source (Morton *et al.*, 1999).

In order to identify additional potential haemoglobin-utilization proteins an affinity isolation protocol was used with haemoglobin as the primary ligand. The affinity isolation protocol was performed on strain HI1714, a derivative of strain HI689 with complete deletion of *hgpA* and *hgpB* and thus retaining expression of only HgpC (Morton *et al.*, 1999). Strain HI1714 was selected in order to

Fig. 1. SDS-PAGE (7.5%) gel stained with Coomassie blue. Lanes: A, affinity-purified outer-membrane proteins from the *H. influenzae* *hgpA/hgpB* double mutant strain HI1714; B, BenchMark Protein Ladder (Invitrogen). Arrows point to: the 120 kDa band corresponding to HgpC; an approximately 105 kDa affinity purified band; an approximately 80 kDa affinity purified band. Molecular masses of the BenchMark protein ladder are from top to bottom 220, 160, 120, 100, 90, 80, 70, 60 and 50 kDa.

use HgpC as an internal control for the affinity isolation procedure; the Hgp proteins are efficiently purified in the haemoglobin affinity isolation protocol (Jin *et al.*, 1999; Ren *et al.*, 1998).

Strain HI1714 yielded two major bands of approximately 120 kDa and 105 kDa and three minor bands (one at approximately 80 kDa and two in the 150–160 kDa range) in the affinity isolation protocol (Fig. 1). The 120 kDa protein corresponded, based on size, to HgpC, and was the most prominent band. The four additional purified bands were submitted for N-terminal amino acid sequencing. The two high-molecular-mass minor proteins yielded no usable amino acid sequence. The 80 kDa protein yielded an amino acid sequence of GQVITIGNERFR(X)PETLFQP, which was determined using BLASTP and TBLASTN searches (www.ncbi.nlm.nih.gov/blast) to be 100% homologous to an internal fragment of actin; no homologous gene products were identified in any available *H. influenzae* genomic sequence and the 80 kDa protein may represent a contaminant derived from the growth medium.

The approximately 105-kDa protein yielded an amino acid sequence of EETLGIDVVEKISNDKKP. This experimentally determined N-terminal amino acid sequence was identical to the processed amino terminus encoded by the ORF designated HI1217 from the *H. influenzae* Rd KW20 genome sequence (Fleischmann *et al.*, 1995). The predicted product of HI1217 from Rd KW20 consists of 886 amino acids

preceded by a 27-residue leader or signal peptide. The molecular mass of the mature protein was calculated to be 99 878 Da. The product of the ORF HI1217 was originally designated a putative transferrin-binding protein (Fleischmann *et al.*, 1995); however, this designation is unlikely to be accurate. Strain Rd KW20 contains an operon (HI0994 and HI0995) that encodes the well-characterized two-protein complex (TbpA and TbpB) involved in transferrin acquisition by *H. influenzae* (Fleischmann *et al.*, 1995; Gray-Owen & Schryvers, 1996), and there is no evidence for multiple transferrin-acquisition pathways in *H. influenzae*. In addition, the protein putatively encoded by HI1217 from Rd KW20 exhibits only 17.3% identity to TbpA (encoded by HI0995) of Rd KW20, while TbpA proteins of five additional *H. influenzae* strains exhibit 92.9 to 94.9% identity with TbpA of Rd KW20 (Loosmore *et al.*, 1996). However, HI1217 contains sequences homologous to conserved domains associated with TonB-dependent proteins and iron-acquisition proteins as determined using the Conserved Domain Database (CDD v. 1.63) (www.ncbi.nlm.nih.gov/structure/cdd/cdd.shtml) (Marchler-Bauer *et al.*, 2003). In all subsequent discussions the ORF HI1217 and its homologues are referred to as *hup* (haem-utilization protein).

Since the predicted product of *hup* has significant homology to other bacterial iron- and/or haem-acquisition-associated proteins and was purified in a haemoglobin affinity purification protocol, we cloned and determined the DNA sequence of the *hup* homologue from strain HI689. The sequence data revealed an encoded protein of 919 amino acids with a 27 amino acid leader sequence predicted using SignalP 2.0 (www.cbs.dtu.dk/services/SignalP). The mature protein of 892 amino acids had a predicted molecular mass of 100 990 Da.

Using the sequence alignment application Align X of the Vector NTI suite v. 8 (Informax), protein sequence alignments between the mature proteins encoded by *hup* in strains HI689 and Rd KW20 showed 90.3% similarity and 88.5% identity. The *hup* gene homologue from type b strain E1a was also cloned and sequenced; analysis of the gene revealed that it encodes a mature protein of 913 amino acids with a predicted molecular mass of 102 800 Da with 90% similarity and 89% identity to the strain HI689 homologue.

Additional *hup* sequences are available from two recently sequenced nontypable *H. influenzae* strains. The gene from strain HI3224A (www.micro-gen.ouhsc.edu) encodes a mature protein of 891 amino acids with a molecular mass of 100 500 Da, and 90.9% similarity and 88.8% identity to the homologous strain HI689 protein; that from strain R2846 (www.genome.washington.edu/uwgc/) encodes a protein of 907 amino acids and 102 000 Da, with 89.4% similarity and 87.9% identity to the HI689 protein.

Hup homologues thus are widespread across the species and show high levels of conservation at the amino acid level.

Such a widespread and highly conserved outer-membrane protein may represent a potential vaccine candidate for prevention of *H. influenzae* disease.

The predicted protein is significantly homologous to putative TonB-dependent outer-membrane receptors from other bacterial species. One such protein from *Neisseria meningitidis* is designated TdfH (TonB-dependent family protein) (Turner *et al.*, 2001). TdfH was identified based on homology to known TonB-dependent proteins including the haemophore receptor HasR of *Serratia marcescens*; however, analysis of an isogenic *tdfH* mutant and expression of the protein in an *Escherichia coli hemA* mutant failed to demonstrate any role for the protein in haem utilization (Turner *et al.*, 2001). TdfH sequences from three *N. meningitidis* strains show approximately 63 % similarity and 51 % identity to Hup from HI689. Additional homologous proteins derived from available bacterial genome sequences are from *Neisseria gonorrhoeae* (64 % similarity, 52 % identity), *Actinobacillus actinomycetem-comitans* (68 % similarity, 56 % identity), *H. somnus* (45 % similarity, 33 % identity) and *Campylobacter jejunii* (42 % similarity, 31 % identity). Although each of these proteins is homologous to TonB-dependent receptors, no function(s) have been experimentally determined for any of them.

Growth characteristics of *hup* deletion mutants

Since *hup* was isolated in a haemoglobin affinity purification protocol and showed significant homology to other putative iron/haem-acquisition-related proteins, the potential role of the protein in haemoglobin utilization was investigated. A complete *hup* deletion mutant of strain HI689 was constructed and designated HI1737. Strains HI689 and HI1737 were compared in growth studies for their ability to utilize haemoglobin as a sole haem source. No statistically significant differences in growth rate or in the final density of the culture were seen between the two strains at haemoglobin concentrations of 1 µg ml⁻¹ and 2 µg ml⁻¹ (data not shown). These results were not surprising since we have previously demonstrated a role for the Hgp proteins of *H. influenzae* in the utilization of haem from haemoglobin (Morton *et al.*, 1999). In view of this role of the Hgp proteins in the utilization of haemoglobin a complete *hup* deletion mutation was constructed in a previously constructed derivative of strain HI689 lacking the *hgp* genes (Morton *et al.*, 1999).

The *hgp* deletion strain (HI1717) and the *hgp hup* deletion mutant (HI1738) were compared for their ability to utilize haemoglobin. At both 1 µg haemoglobin ml⁻¹ and 2 µg haemoglobin ml⁻¹ strain HI1738 consistently exhibited a significantly reduced ability to utilize haemoglobin compared to strain HI1717 (Fig. 2). At higher concentrations of haemoglobin (5 µg ml⁻¹) no significant difference in growth was seen (data not shown). A derivative of the *hgp hup* mutant strain complemented by a plasmid-encoded copy of *hup* (strain HI1953) was also compared to the wild-type strain for growth in 1 µg haemoglobin ml⁻¹: strain

Fig. 2. Growth of the *hgpABC* triple mutant strain HI1717 and the *hgpABC hup* quadruple mutant strain HI1738 in hdBHI supplemented with haemoglobin. Results are for HI1717 in 1 µg haemoglobin ml⁻¹ (●), HI1738 in 1 µg haemoglobin ml⁻¹ (■), HI1717 in 2 µg haemoglobin ml⁻¹ (○) and HI1738 in 2 µg haemoglobin ml⁻¹ (□). Results are mean ± SD for triplicate results from a representative experiment. Using the Kruskal-Wallis test over the entire growth period to compare strains HI1717 and HI1738: at 1 µg haemoglobin ml⁻¹, $P < 0.0001$; at 2 µg haemoglobin ml⁻¹, $P = 0.0032$.

HI1953 grew as well as the wild-type strain HI689 in 1 µg haemoglobin ml⁻¹ ($P = 0.9918$ for one experiment, $P = 0.9506$ for a second experiment). The *hgp hup* mutant strain carrying the plasmid vector alone was unaltered in its ability to utilize haemoglobin (data not shown).

Hup may represent a high-affinity haemoglobin-acquisition system. The effect of deletion of *hup* is not significant at high haemoglobin concentrations, indicating that an additional low-affinity haemoglobin-utilization system may exist. Mutants lacking the Hgp proteins and/or Hup retain the ability to bind haemoglobin in a dot-blot binding assay (data not shown), supporting the hypothesis that additional specific haemoglobin-binding proteins may be present. However, the LPS of other members of the *Pasteurellaceae* and the *Enterobacteriaceae* bind haemoglobin (Belanger *et al.*, 1995; Grenier *et al.*, 1997; Jürgens *et al.*, 2001), although this binding has not been shown to be involved in a haemoglobin-utilization pathway. The LPS of *H. influenzae* may be responsible for the residual haemoglobin binding.

While performing growth studies with haemoglobin as the haem source, growth was also monitored with 10 µg haem ml⁻¹ as a positive control. The *hgp hup* deletion mutant, HI1738, showed a consistent and reproducible delay in the onset of growth as compared to the *hgp* mutant HI1717, with the difference in growth between HI1717 and HI1738 being statistically significant between 6 and 11 h in two independent experiments ($P = 0.0162$ for one experiment, $P = 0.0247$ for a second experiment). This observation led us to further examine the impact of the deletion of *hup* on the utilization of haem.

Fig. 3. Growth of the *hgpABC* triple mutant strain HI1717 and the *hgpABC hup* quadruple mutant strain HI1738 in hdBHI supplemented with haem. Results are for HI1717 in 5 µg haem ml⁻¹ (■), HI1717 in 2 µg haem ml⁻¹ (□), HI1738 in 5 µg haem ml⁻¹ (●) and HI1738 in 2 µg haem ml⁻¹ (○). Results are mean ± SD for triplicate results from a representative experiment. Using the Kruskal–Wallis test over the entire growth period to compare strains HI1717 and HI1738: at 5 µg haem ml⁻¹, $P=0.0003$; at 2 µg haem ml⁻¹, $P<0.0001$.

The *hgp hup* deletion mutant grew significantly less well than the *hgp* mutant HI1717 in limiting levels of haem (Fig. 3). In addition, the single *hup* mutant strain HI1737 grew significantly less well than the wild-type strain HI689 in both 5 µg haem ml⁻¹ and 2 µg haem ml⁻¹ (Fig. 4). However, in contrast to the delayed growth of strain HI1738 as compared to strain HI1717, strain HI1737 grew as well as the wild-type strain in 10 µg haem ml⁻¹ (Fig. 4). Comparison of a complemented *hup* mutant strain (HI1955) with the wild-type strain and the *hup* mutant strain HI1737 for growth in 2 µg haem ml⁻¹ demonstrated that the growth defect was at least partially restored by the presence of *hup* in trans (Fig. 4). Although the complemented strain HI1955 consistently grew to a lower final OD₆₀₅ than the wild-type strain, the difference in growth over the entire curve was not statistically significant ($P=0.081$ for the experiment shown in Fig. 4b, $P=0.2159$ and $P=0.0705$, respectively, for two independent experiments). In contrast the complemented strain HI1955 grew significantly better than the *hup* mutant strain HI1737 ($P<0.0001$ for the experiment shown in Fig. 4, $P=0.0005$ and $P=0.0092$, respectively, for two independent experiments). Strain HI1737 containing the plasmid vector pSU2718 was unaltered in its growth characteristics (data not shown).

The lower final OD₆₀₅ consistently reached by HI1955 as compared to the wild-type strain may result from toxicity of Hup in increased copy number or alternatively from toxicity due to rapid accumulation of haem. However, growth in haemoglobin of an *hgp hup* mutant complemented with *hup* in multicopy did not result in a similar reduction in final OD₆₀₅ compared to the wild-type. Complementation of *hup* with a single chromosomal copy elsewhere in the

Fig. 4. (a) Growth of the wild-type strain HI689 and the *hup* single mutant strain HI1737 in hdBHI supplemented with haem. Results are for HI689 in 10 µg haem ml⁻¹ (○), HI689 in 5 µg haem ml⁻¹ (■), HI689 in 2 µg haem ml⁻¹ (▲), HI1737 in 10 µg haem ml⁻¹ (□), HI1737 in 5 µg haem ml⁻¹ (●) and HI1737 in 2 µg haem ml⁻¹ (×). Results are mean ± SD for triplicate results from a representative experiment. Using the Kruskal–Wallis test: for HI689 versus HI1737 at 10 µg haem ml⁻¹ over the entire growth curve, $P=0.9059$; for HI689 versus HI1737 at 5 µg haem ml⁻¹ over the entire growth curve, $P=0.11$, and from 6.5 h to 24 h, $P=0.0204$; for HI689 versus HI1737 at 2 µg haem ml⁻¹ over the entire growth curve, $P<0.0001$. (b) Growth of the wild-type strain HI689 (▲), the *hup* single mutant strain HI1737 (×) and the *hup* mutant strain harbouring the *hup*-bearing plasmid pDJM367 (strain HI1955; ◇) in hdBHI supplemented with 2 µg haem ml⁻¹. Results are mean ± SD for triplicate results from a representative experiment. Using the Kruskal–Wallis test: for HI689 versus HI1955, $P=0.081$; for HI689 versus HI1737, $P<0.0001$; for HI1955 versus HI1737, $P<0.0001$.

chromosome might resolve this question; however, repeated attempts to construct such a strain have failed. Although it could not be statistically verified, we consistently observed an apparently faster initial rate of growth of strain HI1955 as compared to the wild-type strain HI689, possibly indicating more rapid accumulation of haem.

These data indicate that Hup is a component of a high-affinity haem-acquisition system. Deletion of *hup* alone had no effect on growth in high levels of haem (Fig. 4), while growth at low haem levels was significantly impacted, indicating that an additional low-affinity uptake

mechanism(s) for haem exists. This may explain why mutation of the *hup* homologue in *N. meningitidis* (TdfH) was reported to have no effect on haem utilization (Turner *et al.*, 2001); studies in limiting haem may demonstrate a role for TdfH in haem utilization. Comparison of the data for the impact of deletion of *hup* alone and combined with an *hgp* deletion at high levels of haem indicate a possible role for the Hgp proteins in haem acquisition; however, it is possible that the delay in onset of growth of the *hgp/hup* deletion mutant (HI1738) results from a generally less robust organism rather than a specific effect of *hgp* deletion on haem acquisition. Comparison of the growth of strains HI689 and HI1717 in limiting levels of haem (Figs 3 and 4) demonstrated that the triple *hgp* deletion mutant (HI1717) grew significantly less well than the wild-type ($P=0.0197$ at $5 \mu\text{g haem ml}^{-1}$), similarly indicating a potential role for the Hgp proteins in haem utilization that warrants further investigation.

In view of the demonstrated role of Hup in the utilization of both haem and haemoglobin, its potential role in the utilization of additional haem sources known to be used by *H. influenzae in vitro* (haem–haemopexin, haem–albumin and haemoglobin–haptoglobin) (Stull, 1987) was investigated. While the wild-type strain utilized haem–haemopexin as a haem source, the *hup* deletion mutant strain was unable to grow with haem–haemopexin as the sole haem source (Fig. 5). Similarly the *hup* deletion mutant was unable to utilize haemoglobin–haptoglobin ($1 \mu\text{g haemoglobin equivalent ml}^{-1}$) as a haem source (Kruskal–Wallis test over entire growth curve comparing HI689 and HI1737 $P<0.0001$) (data not shown). Growth of the *hup* deletion mutant with haem–albumin as the sole haem source was significantly delayed as compared to the wild-type strain; however, the mutant strain eventually grew to the same bacterial density as the wild-type strain (Fig. 6).

Fig. 5. Growth of the wild-type strain HI689 (■) and the *hup* single mutant strain HI1737 (□) in hdBHI supplemented with haem–haemopexin at $200 \text{ ng haem equivalent ml}^{-1}$. Results are mean $\pm$ SD for triplicate results from a representative experiment. Using the Kruskal–Wallis test over the entire growth curve $P<0.0001$.

Fig. 6. (a) Growth of the wild-type strain HI689 (■) and the *hup* single mutant strain HI1737 (□) in hdBHI supplemented with haem–albumin at $1 \mu\text{g haem equivalent ml}^{-1}$. Results are mean $\pm$ SD for triplicate results from a representative experiment. Using the Kruskal–Wallis test over the entire growth curve, $P<0.0001$. (b) Growth of the wild-type strain HI689 (■), the wild-type strain HI689 after prior passage through haem–albumin (□) and the *hup* single mutant strain HI1737 after prior passage through haem–albumin (●) in hdBHI supplemented with haem–albumin at $1 \mu\text{g haem equivalent ml}^{-1}$. Results are mean $\pm$ SD for triplicate results from a representative experiment. Using the Kruskal–Wallis test over the entire growth curve none of the differences between these data were statistically significant.

Stationary-phase organisms from the *hup* mutant growth curve in haem–albumin were recovered and their antibiotic-resistance profile confirmed. These recovered organisms were subsequently compared to the wild-type strain for growth in haem–albumin (Fig. 6). The mutant strain (HI1737) previously passed through haem–albumin was indistinguishable in its growth in haem–albumin from the wild-type strain. Additionally, the complemented strain HI1955 grew as well as the wild-type strain ($P=0.7863$ and $P=0.2141$ for two independent experiments) and significantly better than the *hup* mutant strain HI1737 ($P=0.0061$ and $P=0.0038$ for two independent experiments) with haem–albumin as the sole haem source.

The initially observed growth difference between the *hup* deletion mutant and the wild-type strain demonstrate a role for Hup in the utilization of haem–albumin. However, the eventual growth of the mutant indicates an additional

Hup-independent pathway for acquisition of haem from haem–albumin. The growth of the haem–albumin-passaged *hup* deletion mutant at wild-type levels suggests that the alternative haem–albumin-acquisition pathway may be phase variable; a similar phenomenon is observed with growth in haemoglobin–haptoglobin and is attributable to one or more of the Hgp proteins shifting from an unexpressed state to an expressed state due to a frame alteration caused by strand slippage across a length of CCAA nucleotide repeats in the gene (Morton *et al.*, 1999; Ren *et al.*, 1999). The change in haem–albumin phenotype is not due to alteration in expression of the Hgp proteins, since growth of the *hgp hup* quadruple mutant gives the same results as reported above for the *hup* single mutant (data not shown).

It should be noted that the delay in onset of growth in the *hup* mutant strain varies widely in length between independent experiments; we have seen delays varying from 3 h up to 10 h, possibly reflecting the percentage of cells expressing the putative phase-variable moiety in a given inoculum. No candidate phase-variable genes were identified in the available *H. influenzae* genomic sequences; however, the moiety responsible for the haem–albumin growth phenomenon observed in HI689 may not be present in the sequenced strains. Thus, it is not possible to identify the additional haem–albumin-acquisition pathway at this time.

The precise role of Hup in the acquisition of haem cannot be deduced at this time, although its apparent role in the acquisition of haem from several haem sources indicates that it may act as part of a central pathway through which haem from various sources is internalized. Whether Hup acts as a specific outer-membrane receptor for any particular haem source is not clear; although we initially isolated Hup in a haemoglobin affinity protocol this may reflect more an affinity for the haem moiety rather than specific binding of haemoglobin. Whole-cell haemoglobin-binding assays with the *hup* and *hup hgp* deletion mutants have not resolved this issue.

The failure of the *hup* deletion mutant to grow with haemoglobin–haptoglobin indicates that Hup may act as an accessory protein for the Hgp proteins, possibly by facilitating removal of haem from the protein complex or transporting haem across the outer membrane. The impact of deletion of *hup* on haem–haemopexin-utilization indicates a possible interaction between HxuC and Hup since *hxuC* mutations also result in an inability of the mutant to grow on haem–haemopexin (Cope *et al.*, 1995). The possible interaction between Hup and HxuC is further supported by the failure of both an *hup* mutant and an *hxuC* mutant (Cope *et al.*, 1995) to grow on low levels of free haem. Similarly both *hup* and *hxuC* (Cope *et al.*, 2001) mutant strains are deficient in the ability to utilize haemoglobin when the *hgp* genes have also been deleted. Haem–albumin-utilization is also impacted by mutations in either *hup* or *hxuC*, although non-Hup-mediated

acquisition mechanisms for this haem source also exist. These data point to a central role for Hup in haem acquisition by *H. influenzae*, possibly in association with HxuC and/or the Hgp proteins. We hypothesize that Hup is an accessory protein for specific outer-membrane protein receptors to remove haem from various haem-binding proteins. Additional work is under way to clarify the role of Hup and to define its interactions with other haem-acquisition proteins and/or pathways.

Contribution of Hup to virulence in the infant rat model of invasive infection

To determine whether Hup contributes significantly to the virulence of *H. influenzae* in the infant rat model of invasive disease, a cohort of 5-day-old rat pups was infected with the wild-type strain HI689, the *hup* deletion mutant (HI1737), the *hgp* deletion mutant (HI1717) or the *hup/hgp* mutant (HI1738). At an infective dosage of 200 c.f.u., each of the strains was highly effective in establishing bacteraemia. All animals (10/10) infected with each strain became bacteraemic within 24 h after infection. The antibiotic-resistance profile of recovered organisms was determined to ensure that the infecting organism was the correct strain.

The magnitudes of bacteraemia produced by the wild-type strain HI689 and the *hup* deletion strain HI1737 were indistinguishable (data not shown). Although both the *hgp* deletion strain (HI1717) and the *hup/hgp* deletion mutant (HI1738) exhibited a trend towards a reduction in bacteraemic titre on days 1 to 3 post-infection, none of the bacteraemic titres produced by these strains differed in a statistically significant manner from those produced by challenge with the wild-type strain. Clearance of bacteraemia also did not differ among rat pups infected with the different strains; no infant rats cleared their bacteraemia over the 7-day period of this experiment. Similarly the clinical effect of all *H. influenzae* strains was indistinguishable. All animals had significant tremor 3 to 5 days post-infection (an indication of meningitis) but none was hypothermic to touch, exhibited anorexia, had a slow righting reflex or showed depressed locomotor activity.

These data indicate that expression of Hup is not required for establishment and maintenance of infection in the infant rat model of invasive disease. Since neither the Hgp proteins nor Hup are necessary for infection in this model it is unlikely that either haemoglobin–haptoglobin or haem–haemopexin are necessary haem sources in this model of invasive disease. It is possible that the *H. influenzae* haemoglobin–haptoglobin and/or haem–haemopexin uptake mechanisms are highly specific for the haem-binding proteins from the natural host species; if *H. influenzae* is unable to utilize haemoglobin–haptoglobin or haem–haemopexin from the rat then mutation of the acquisition pathways would not be expected to affect pathogenicity in this model.

No data are available on the relative ability of *H. influenzae* to utilize haemoglobin–haptoglobin complexes from

different mammalian sources; *H. influenzae* can utilize haem-haemopexin complexes from both humans and rabbits (Wong *et al.*, 1994), but no data are available with respect to rat haemopexin. It is clear from growth studies reported above that either haemoglobin or haem-albumin can be utilized by the *hup* and the *hup hgp* mutants *in vitro*; either haemoglobin or haem-albumin may represent a significant haem source during invasive disease in the 5-day old infant rat. *H. influenzae* grows well *in vitro* on haemoglobin from a wide range of mammalian sources including the rat, but it is not known if *H. influenzae* can utilize rat haem-albumin complexes as an *in vitro* haem source. Studies on the relative abilities of *H. influenzae* to utilize haem sources from various mammalian sources would be of potential value in analysing *in vivo* data.

We have recently shown that an Hgp deletion mutant of a nontypable strain has reduced virulence in a chinchilla model of otitis media (Morton *et al.*, 2004), in contrast to the Hgp deletion mutant of a type b strain in the infant rat. That report indicates that haemoglobin-haptoglobin may be a primary source of haem in the middle ear of the chinchilla with experimentally induced otitis media. These data illustrate that both the complexity of the *H. influenzae* haem-acquisition systems and the potential for variable haem sources at different infection sites in the host render studies on the *in vivo* impact of mutations in the *H. influenzae* haem-acquisition pathways difficult. The findings reported here with respect to the lack of importance of Hup in the infant rat model of invasive disease do not preclude its relevance in other sites of disease and/or colonization.

In summary, the newly identified haem-utilization protein, Hup, may be an important cofactor in mediating haem acquisition from multiple human haem sources.

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