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
by the serum proteins haemopexin and albumin and cleared from the circulation (Genco & Dixon, 2001; Peters, 1996). Haemoglobin and the haemopexin–haptoglobin, haem–haemopexin and haem–albumin complexes, as well as PPIX in the presence of an iron source such as ferritin, 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 *huxCA* 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 ferritin in 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/haemopexin–haptoglobin-binding proteins (*hgpA*, *hgpB* and *hgpC* in the type b strain Hl689) (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 Hl689 is a clinical isolate and has been previously described (Jin *et al.*, 1996; Musser *et al.*, 1986). Strain Hl1717 is a derivative of strain Hl689 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 containing bacitracin (BBL) at 37°C. When necessary, *H. influenzae* was grown on brain heart infusion (BHI) agar (Difco) complemented 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 haemoglobin 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 haemoglobin 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 Hl689.** 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 Hl1217 locus. 1217A and 1217REV/SQ2 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* Hl689 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/1217REV/SQ2 and 57°C for primer pair 1217SQ2/1217SQ101, and primer extension at 72°C for 1 min, with
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* sites 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 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 1. Strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)*</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td><strong>Strains</strong> <em>H. influenzae</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HI689</td>
<td>Type b</td>
<td>Musser et al. (1986)</td>
</tr>
<tr>
<td>HI1714</td>
<td>HI689, <em>ΔhgpA ΔhgpB Rb' Tc'</em></td>
<td>Morton et al. (1999)</td>
</tr>
<tr>
<td>HI1717</td>
<td>HI689, <em>ΔhgpA ΔhgpB ΔhgpC Rb' Tc' Sp'</em></td>
<td>Morton et al. (1999)</td>
</tr>
<tr>
<td>HI1737</td>
<td>HI689 <em>Δhup, Zeo'</em></td>
<td>This study</td>
</tr>
<tr>
<td>HI1738</td>
<td>HI1717 <em>Δhup, Zeo'</em></td>
<td>This study</td>
</tr>
<tr>
<td>HI1955</td>
<td>HI1737, pDJM367, <em>Zeo'</em> <em>Cmr'</em></td>
<td>This study</td>
</tr>
<tr>
<td>HI1953</td>
<td>HI1738, pDJM367, <em>Rbc' Tc' Sp' Zeo' Cmr'</em></td>
<td>This study</td>
</tr>
<tr>
<td>Ela</td>
<td>Type b</td>
<td>Stull et al. (1984)</td>
</tr>
<tr>
<td><strong>E. coli</strong> TOP10</td>
<td>Used for cloning in pCR2.1-TOPO and pUC19N</td>
<td>Invitrogen</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pCR2.1-TOPO</td>
<td>pUC19 with a NotI site added at the HindIII end of the polylinker</td>
<td>Tartof &amp; Hobbs (1988)</td>
</tr>
<tr>
<td>pUC19N</td>
<td><em>E. coli</em>-<em>H. influenzae</em> shuttle vector, lacZa, Cmr, p15a ori</td>
<td>Martinez et al. (1988)</td>
</tr>
<tr>
<td>pSU2718</td>
<td>Plasmid expressing Zeo' (<em>Sh ble</em>) using the bacterial EM7 promoter; the Zeo' cassette is flanked by polylinkers to allow excision of the resistance marker</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pEM7/Zeo</td>
<td>pUC19N carrying an ~1100 bp PCR product corresponding to a region upstream of <em>hup</em> and an ~1100 bp product corresponding to a region downstream of <em>hup</em> with a unique <em>BamHI</em> site between the two inserts</td>
<td>This study</td>
</tr>
<tr>
<td>p1217UD-ZEO</td>
<td>p1217UD with the ~700 bp <em>Zeo</em> marker from pEM7/Zeo inserted at the unique <em>BamHI</em> site</td>
<td>This study</td>
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<tr>
<td>pDJM359</td>
<td>pCR2.1-TOPO carrying an ~3200 bp PCR product encompassing the <em>hup</em> coding sequence and upstream and downstream regions</td>
<td>This study</td>
</tr>
<tr>
<td>pDJM367</td>
<td>pSU2718 carrying the 3200 bp PCR product from pDJM359</td>
<td>This study</td>
</tr>
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</table>

*Amp*, ampicillin resistance (50 μg ampicillin ml⁻¹ in *E. coli*); *Cmr*, chloramphenicol resistance (1-5 μg chloramphenicol ml⁻¹ in *H. influenzae* and 50 μg chloramphenicol ml⁻¹ in *E. coli*); Kan', kanamycin resistance (50 μg kanamycin ml⁻¹ in *E. coli*); Sp', spectinomycin resistance (200 μg spectinomycin ml⁻¹ in *H. influenzae*); *Tc*, tetracycline resistance (3 μg tetracycline ml⁻¹ in *H. influenzae*); *Zeo', zeocin resistance (50 μg zeocin ml⁻¹ in *H. influenzae* and 100 μg zeocin ml⁻¹ in *E. coli*).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’–3’).</th>
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<tbody>
<tr>
<td>1217REV5Q2</td>
<td>CTCAAGACGATGTGCTAGG</td>
</tr>
<tr>
<td>1217A</td>
<td>GTTCGACGATTCTGAGG</td>
</tr>
<tr>
<td>1217SQ2</td>
<td>TAGGACATGGTCGGG</td>
</tr>
<tr>
<td>1217SQ101</td>
<td>CTTCTTTACTGATCCG</td>
</tr>
<tr>
<td>1217-1</td>
<td>GAGTTCCATTACCATTATTATTATTAGAGG</td>
</tr>
<tr>
<td>1217-2</td>
<td>CGTCCAGCTTTTTTTTTTTCTGATGG</td>
</tr>
<tr>
<td>1217-3</td>
<td>GAGTTCCATGGTAATGCTATGC</td>
</tr>
<tr>
<td>1217-4</td>
<td>GAATTCCGATACCTGGTACTAGG</td>
</tr>
<tr>
<td>1217-5</td>
<td>GAATTCCCTAGTACCTTTATATAC</td>
</tr>
<tr>
<td>1217-6</td>
<td>GAATTCCTTAGTACCTTTATATATA</td>
</tr>
</tbody>
</table>

*Downloaded from www.microbiologyresearch.org by 54.70.40.11 On: Mon, 05 Aug 2019 16:17:43*
was excised by digestion with BamHI and BglII and cloned into the unique BamHI 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 \(\mu\)g zeocin ml\(^{-1}\), with subsequent subculture of potential transformants at 50 \(\mu\)g 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 \(^\circ\)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 \(KpnI\) and \(SphI\) and the band corresponding to the chromosomally derived insert was ligated to \(KpnI/SphI\)-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 \(\mu\)g 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 at 37 \(^\circ\)C with shaking (175 r.p.m.). Growth was monitored to the stationary phase by measurement of the OD\(_{605}\) with a Shimadzu spectrophotometer. Stationary phase cultures were used to inoculate fresh hdBHI (0-1% inoculum to give an approximate initial concentration of 200 000 c.f.u. ml\(^{-1}\)). These broth cultures were incubated at 37 \(^\circ\)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 OD\(_{605}\) of 0-5 and then diluted serially in the same solution to provide the standard inoculum (200 c.f.u. in 100 \(\mu\)l) that was injected intraperitoneally into 5-day-old rat pups. To determine the actual infective dosage, 100 \(\mu\)l 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 \(\mu\)l) 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 \(\mu\)l from each dilution were plated in triplicate on chocolate agar plus bacitracin and incubated at 37 \(^\circ\)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 and 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...
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 H13224A (www.micro-gen.oulhsc.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/ugwc/) 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 actinomycetemcomitans* (68% similarity, 56% identity), *H. somnus* (45% similarity, 33% identity) and *Campylobacter jejuni* (42% similarity, 31% identity). Although some of these proteins are 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 *hup* 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 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.
The \textit{hgp} \textit{hup} deletion mutant grew significantly less well than the \textit{hgp} mutant \textit{HI1717} in limiting levels of haem (Fig. 3). In addition, the single \textit{hup} mutant strain \textit{HI1737} grew significantly less well than the wild-type strain \textit{HI689} in both 5 \(\mu\)g haem ml\(^{-1}\) and 2 \(\mu\)g haem ml\(^{-1}\) (Fig. 4). However, in contrast to the delayed growth of strain \textit{HI1738} as compared to strain \textit{HI1717}, strain \textit{HI1737} grew as well as the wild-type strain in 10 \(\mu\)g haem ml\(^{-1}\) (Fig. 4).

Comparison of a complemented \textit{hup} mutant strain (\textit{HI1955}) with the wild-type strain and the \textit{hup} mutant strain \textit{HI1737} for growth in 2 \(\mu\)g haem ml\(^{-1}\) demonstrated that the growth defect was at least partially restored by the presence of \textit{hup} in trans (Fig. 4). Although the complemented strain \textit{HI1955} consistently grew to a lower final \(OD_{605}\) 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 \textit{HI1955} grew significantly better than the \textit{hup} mutant strain \textit{HI1737} (\(P<0.0001\) for the experiment shown in Fig. 4, \(P=0.0005\) and \(P=0.0092\), respectively, for two independent experiments). Strain \textit{HI1737} containing the plasmid vector \textit{pSU2718} was unaltered in its growth characteristics (data not shown).

The lower final \(OD_{605}\) consistently reached by \textit{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 of haemoglobin of an \textit{hgp} \textit{hup} mutant complemented with \textit{hup} in multicopy did not result in a similar reduction in final \(OD_{605}\) compared to the wild-type. Complementation of \textit{hup} with a single chromosomal copy elsewhere in the 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 \textit{HI1955} as compared to the wild-type strain \textit{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 \textit{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 system become limiting in the mutant.
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 \((P=0.0197\text{ at }5\mu g\text{ haem ml}^{-1})\), 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 µg 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).

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 passaged 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 \((P=0.7863\text{ and }P=0.2141\text{ for two independent experiments})\) and significantly better than the hup mutant strain HI1737 \((P=0.0061\text{ and }P=0.0038\text{ for two independent experiments})\) 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 hxcC 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 hxcC mutant (Cope et al., 1995) to grow on low levels of free haem. Similarly both hup and hxcC (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 hxcC, 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 bacteremic 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 significantly manner from those produced by challenge with the wild-type strain. Clearance of bacteremia also did not differ among rat pups infected with the different strains; no infant rats cleared their bacteremia 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 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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