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’Brien, 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 reticuloendothelial 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 and
haemoglobin and the haem–haemopexin–haptoglobin, haem–haemopexin and haem–albumin complexes, as well as PPIX
in the presence of an iron source such as ferrittransferrin, 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 hxuCBA 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. Van Wagener, 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 Hl689 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 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 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; BHIB) 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 polyvinylene 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. 127A 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

<table>
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<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)*</th>
<th>Reference or source</th>
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<td><strong>Strains</strong></td>
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<tr>
<td>H. influenzae</td>
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<tr>
<td>H1689</td>
<td>Type b</td>
<td>Musser et al. (1986)</td>
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<tr>
<td>H11714</td>
<td>H1689, ΔhgpA ΔhgpB, Rb', Tc'</td>
<td>Morton et al. (1999)</td>
</tr>
<tr>
<td>H11717</td>
<td>H1689, ΔhgpA ΔhgpB ΔhgpC, Rb' Tc'</td>
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<tr>
<td>H11737</td>
<td>H1689 Δhup, Zeo'</td>
<td>This study</td>
</tr>
<tr>
<td>H11738</td>
<td>H11717 Δhup, Zeo'</td>
<td>This study</td>
</tr>
<tr>
<td>H11955</td>
<td>H11737, pDJM367, Zeo' Cm'</td>
<td>This study</td>
</tr>
<tr>
<td>H11953</td>
<td>H11738, pDJM367, Rb' Tc' Sp' Zeo' Cm'</td>
<td>This study</td>
</tr>
<tr>
<td>Ela</td>
<td>Type b</td>
<td>Stull et al. (1984)</td>
</tr>
<tr>
<td>E. coli TOP10</td>
<td>Used for cloning in pCR2.1-TOPO and pUC19N</td>
<td>Invitrogen</td>
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| **Plasmids**      |                                |                     |
| pCR2.1-TOPO       | Plac, lacZz, Kan' Amp', ColE1 origin, F1 origin, T7 promoter |                     |
| pUC19N            | pUC19 with a NotI site added at the HindIII end of the polylinker | Tartof & Hobbs (1988) |
| pSU2718           | E. coli-H. influenzae shuttle vector, lacZz, Cm', p15a ori | Martinez et al. (1988) |
| pEM7/Zeo          | Plasmid expressing Zeo'(Sh ble) using the bacterial EM7 promoter; the Zeo' 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 hup and an ~1100 bp product corresponding to a region downstream of hup with a unique BamHI site between the two inserts | This study |
| p1217UD-ZEO       | p1217UD with the ~700 bp Zeo' marker from pEM7/Zeo inserted at the unique BamHI site | This study |
| pDJM359           | pCR2.1-TOPO carrying an ~3200 bp PCR product encompassing the hup coding sequence and upstream and downstream regions | This study |
| pDJM367           | pSU2718 carrying the 3200 bp PCR product from pDJM359 | This study |

*Amp’, ampicillin resistance (50 μg ampicillin ml⁻¹ in E. coli); Cm’, 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).

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

<table>
<thead>
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<th>Primer</th>
<th>Sequence (5’–3’)</th>
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</tr>
<tr>
<td>1217A</td>
<td>GTTGAGCAGATTTGCTAGGG</td>
</tr>
<tr>
<td>1217SQ2</td>
<td>TAGGAGCTGTTGCTAGGT</td>
</tr>
<tr>
<td>1217SQ101</td>
<td>CTTGTTTACGCACGCC</td>
</tr>
<tr>
<td>1217-1</td>
<td>GGATGCCTATACACATTATTATAGGG</td>
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<tr>
<td>1217-2</td>
<td>CTGCAGGCTTATTATTTCTGATGG</td>
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<tr>
<td>1217-3</td>
<td>GGATCCCGCATAAGCATATGCTAGTGC</td>
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<td>1217-4</td>
<td>GAATTCCGAATAACCGTATTACGG</td>
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<td>1217-5</td>
<td>GAATTCCTAGTACCTTTATTTATAATC</td>
</tr>
<tr>
<td>1217-6</td>
<td>GAATTCCTAGTACCTTTATTTATAATA</td>
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</table>

**Construction of an hup deletion mutant.** Complete deletion of hup in strains H1689 and H11717 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 H1689. 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 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 HI689 abutting each other, with a unique BamHI site engineered between the upstream and downstream regions. The zeocin-resistance marker from pEM7/Zeo.
was diluted in 5 ml of 0.2% (w/v) gelatin in PBS and resuspended to an optical density at 605 nm of 0.1 % (w/v) gelatin. One millilitre of the bacterial suspension was adjusted to an OD605 of 0.1 % (w/v) gelatin, and this final suspension was filtered through 0.45 µm filters before use in inoculation experiments.

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 with 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 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. Electro- poration 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 200000 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 (Haran 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 housed on hardwood litter with ad libitum, and thus retaining expression of only HgpC (Morton et al., 1999). Strain H11714 was selected in order to isolate 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 H1689, a derivative of strain H1689 with complete deletion of hgpA and hgpB and thus retaining expression of only HgpC (Morton et al., 1999).
The 80 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 isolation protocol, we cloned and determined the DNA sequence of the hup homologue from strain H1689. 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 H1689 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 H1689 homologue.

Additional hup sequences are available from two recently sequenced nontypable H. influenzae strains. The gene from strain H13224A (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 H1689 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 H1689 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 receptors 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 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 final density of the culture were seen between the two strains at haemoglobin concentrations of 1 mg haemoglobin 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 mg haemoglobin ml⁻¹ and 2 mg 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 mg 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 mg haemoglobin ml⁻¹: strain HI1953 grew as well as the wild-type strain HI689 in 1 mg 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 mg 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 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 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 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 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 utilize 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).

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

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 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 H1689 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 hxC 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 hxC mutant (Cope et al., 1995) to grow on low levels of free haem. Similarly both hup and hxC (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 hxC, 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 H1689, the hup deletion mutant (H11737), the hgp deletion mutant (H11717) or the hup/hgp mutant (H11738). 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 H1689 and the hup deletion strain H11737 were indistinguishable (data not shown). Although both the hgp deletion strain (H11717) and the hup/hgp deletion mutant (H11738) 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 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–haemopexin 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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