The haem–haemopexin utilization gene cluster (hxuCBA) as a virulence factor of Haemophilus influenzae

Daniel J. Morton,1 Thomas W. Seale,1 Larissa L. Madore,1 Timothy M. VanWagoner,1 Paul W. Whitby1 and Terrence L. Stull1,2

Correspondence
Terrence L. Stull
terrence-stull@ouhsc.edu

1Department of Pediatrics, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, USA
2Department of Microbiology/Immunology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, USA

Haemophilus influenzae has an absolute growth requirement for a porphyrin source, which can be supplied in vitro by haem, haemoglobin, or the haemoglobin–haptoglobin, haem–haemopexin and haem–albumin complexes. Utilization of the haem–haemopexin complex is known to be mediated by the products of the hxuCBA gene cluster. It was demonstrated that hxuC, but not hxuA or hxuB, is also essential for the utilization of haem from haem–albumin complexes. Mutants of the type b strain E1a lacking genes in the hxuCBA genes yielded a significantly reduced bacteraemic titre compared to the wild-type strain. In addition, 5-day-old rats infected with the hxuCBA mutant strains exhibited significantly improved survival rates compared to those infected with the wild-type strain. Mutations in the haemoglobin/haemoglobin–haptoglobin-binding protein genes (hgp)s, either alone or in combination with the hxuCBA mutations, had no impact on virulence in 5-day-old rats. In 30-day-old rats infected with either the hxuCBA mutants or the wild-type strains, there was no significant difference in the ability to establish bacteraemia although bacterial titres were lower in rats infected with the hxuCBA mutants than in those infected with the wild-type strain. These age-related differences in the impact of mutations in the hxuCBA gene cluster may be related to changes in levels of host haem-binding proteins during development of the rat.

INTRODUCTION

Haemophilus influenzae is a fastidious Gram-negative bacterium that is an important cause of human infections including otitis media, meningitis and pneumonia (Turk, 1984). H. influenzae is unable to synthesize protoporphyrin IX (PPIX), the immediate precursor of haem, since it lacks all enzymes in the biosynthetic pathway for the porphyrin ring (Panek & O’Brien, 2002; White & Granick, 1963). However, most H. influenzae strains express a ferrochelatase which mediates insertion of iron into PPix to form haem (Schlor et al., 2000; Panek & O’Brien, 2002; Loeb, 1995). Thus, H. influenzae has an absolute growth requirement for exogenous PPix or a haem source. Since the only known niche for H. influenzae is humans, the organism must adapt its mechanisms of haem acquisition accordingly (Morton & Stull, 2004). Haem is generally intracellular, in the form of haemoglobin or haem-containing enzymes, and unavailable to invading micro-organisms (Genco & Dixon, 2001; Griffiths, 1999). Extracellular 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 (Ward & Bullen, 1999; Evans et al., 1999). Free haem, principally derived from the dissociation of methaemoglobin, is bound by the serum proteins haemopexin and albumin and cleared from the circulation (Peters, 1996; Genco & Dixon, 2001).

Haemoglobin and the haemoglobin–haptoglobin, haem–haemopexin and haem–albumin complexes can all be utilized by H. influenzae as haem sources in vitro (Stull, 1987). We have shown that utilization of haemoglobin–haptoglobin is mediated by a family of phase-variable haemoglobin/haemoglobin–haptoglobin–binding proteins (the Hgps) (Morton et al., 1999). While the Hgp proteins have a role in the utilization of haemoglobin, analysis of hgp null mutants demonstrates that additional pathways are clearly involved in utilization of this haem source (Morton
et al., 1999, 2004b). Utilization of the haem–haemopexin complex is mediated by the products of the hxuCBA gene cluster (Hanson et al., 1992; Cope et al., 1995). HxuA is a 100 kDa secreted protein that binds the haem–haemopexin complex (Cope et al., 1994, 1998). The HxuA–haem–haemopexin complex is presumably bound by the bacterial cell and the haem is internalized; the outer-membrane moiety mediating the binding of HxuA has not been identified although the receptor may be HxuC (Cope et al., 1998). HxuC possesses the amino acid sequences considered characteristic of TonB-dependent proteins that are typically involved in the binding and transport of certain low-molecular-mass ligands (Postle, 1990; Cope et al., 1995).

HxB is believed to be involved in the secretion of the soluble HxuA molecule from the bacterial cell (Cope et al., 1995). Mutations in hxuA and/or hxuB abolish the ability to utilize the haem–haemopexin complex (Cope et al., 1995). HxuC is required for the utilization of both the haem–haemopexin complex and low levels of free haem (Cope et al., 1995); HxuC has also been implicated in the residual utilization of haemoglobin seen in an hgp knockout background (Cope et al., 2001). Transcripts of the gene encoding HxuC can be detected in the human middle ear during acute otitis media (Whitby et al., 1997), and transcription of all three genes in the hxuCBA gene cluster is upregulated under conditions of haem starvation as would be encountered in the human host (Whitby et al., 2006). Both these latter findings suggest that the proteins encoded by the hxuCBA gene cluster are expressed in vivo and may represent important virulence determinants.

The object of this study was to further characterize potential haem sources utilized via the HxuCBA proteins and to elucidate the potential role of the hxuCBA gene cluster in the virulence of invasive H. influenzae infection in infant and weanling rat models.

### 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 E1a is a clinical isolate from cerebrospinal fluid and has been previously described (Stull et al., 1984). 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-depleting growth was performed in BHI broth supplemented with only 10 µg β-NAD ml⁻¹ (haem-depleting BHI; hdBHI). H. influenzae were transfected to antibiotic resistance using a modification of the static aerobic method of Gromkova et al. (1989) as previously described (Morton et al., 2004b).

#### Haem sources

Human serum albumin (HSA), and haemmin were purchased from Sigma. Stock haem solutions were prepared at 1 mg haem ml⁻¹ as previously described (Morton et al., 2005). Haemoglobin was dissolved in water immediately before use. 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 & Morgan, 1984; Smith, 1985).

#### 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 Miniprep 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 Operon Technologies.

#### Sequencing of the hxuCBA gene cluster from H. influenzae strain Eta

To sequence the hxuCBA cluster, four overlapping PCRs were performed and the resulting amplified products were cloned and sequenced. Four separate pairs of primers (Table 2) were designed for use in the PCR based on the Rd KW20 genomic sequence. The PCRs were performed in a 50 µl reaction as previously described (Morton et al., 2004b) except that annealing temperatures and extension times were as below. HXUCUSA and HXUCUSB were used to amplify an ~1100 bp fragment upstream of hxuC (annealing temperature 56°C, extension time 1 min); HXUC-A and HXUC-B to amplify an ~2500 bp fragment encompassing the entire coding sequence of hxuC (annealing temperature 58°C, extension time 2 min 40 s); HXUCDSA and HXUBDSB to amplify an ~2800 bp fragment including the entire coding sequence of hxuB and part of hxuA (annealing temperature 56°C, extension time 3 min); and HXUBDSA and HXUADSB to amplify an ~3000 bp fragment including the entire coding sequence of hxuA and ~1000 bp downstream of hxuA (annealing temperature 60°C, extension time 4 min). PCR products of the appropriate size were cloned into pCR2.1-TOPO and transformed into competent Escherichia coli TOP10. Cloned PCR products from at least two separate PCRs were sequenced for each of the four overlapping fragments.

#### Construction of hxuC and hxuBA deletion mutants

Complete deletion mutants were constructed that lacked either hxuC alone or the region encompassing hxuA and hxuB. These mutants were constructed as described previously (Morton et al., 1999, 2004b). For deletion of hxuC, two pairs of primers were designed to amplify regions upstream and downstream of hxuC respectively (Table 2). Primer pair HXUCUSA and HXUCUSB was used in the PCR as described above and it amplified a 1073 bp product upstream of hxuC with engineered EcoRI and BamHI sites at the ends of the amplicon to allow for directional cloning. The second primer pair, HXUCDSA and HXUCDSB, was designed to amplify a 966 bp product downstream of hxuC in the PCR (annealing at 55°C, extension time 1 min) and similarly add PstI and BamHI sites to the ends of the amplicon. PCR products of the correct size were initially cloned into pCR2.1-TOPO, then sequencially subcloned into pUC19N to yield a plasmid that contained upstream and downstream sequences of hxuC abutting each other with a unique BamHI site between them. This unique BamHI site was employed in the insertion of an ~1200 bp BamHI-excised kanamycin-resistance-encoding marker from pKANR to yield pDM343. Plasmid pKANR was constructed in this laboratory and consists of a Tn903-derived aminoglycoside resistance cassette flanked by multiple paired restriction sites (excisable by EcoRI, BamHI, XbaI, SfiI, SphI and HindIII), and was constructed by performing a PCR using the primers KAN-2-F (5’-GGATCCCTACGATGCACGCGATGATTCGATTGTCGTC- TCAAAAATCTCTGATG-3’) and KAN-2-R (5’-GAGGCTGGATTCGCCTGACGATC-3’) and a random plasmid derived from a H.
**Table 1. Strains and plasmids**

<table>
<thead>
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<th>Strain or plasmid</th>
<th>Relevant characteristic(s)*</th>
<th>Reference or source</th>
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<tbody>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>E1a</td>
<td>Type b</td>
<td>Stull et al. (1984)</td>
</tr>
<tr>
<td>HI1796</td>
<td>E1a, ΔhxuC Kan&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>HI1764</td>
<td>E1a, ΔhxuBA Erm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>HI1758</td>
<td>E1a, ΔhgpB Δhgc-C Sp&lt;sup&gt;+&lt;/sup&gt; Tc&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>HI1797</td>
<td>HI1758, ΔhxuC Kan&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>HI1763</td>
<td>HI1758, ΔhxuBA Erm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOP10</td>
<td>Used for cloning in pCR2.1-TOPO and pUC19N</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCR2.1-TOPO</td>
<td>Plac, lacZs, Kan&lt;sup&gt;+&lt;/sup&gt; Amp&lt;sup&gt;+&lt;/sup&gt;, ColE1 origin, F1 origin, T7 promoter</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pUC19N</td>
<td>pUC19 with a Ncol site added at the HindIII end of the polylinker</td>
<td>Tartof &amp; Hobbs (1988)</td>
</tr>
<tr>
<td>pERMR</td>
<td>pCR2.1-TOPO containing an ~1300 bp erythromycin resistance marker</td>
<td>This study</td>
</tr>
<tr>
<td>pKANR</td>
<td>pCR2.1-TOPO containing an ~1200 bp kanamycin resistance marker</td>
<td>This study</td>
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<tr>
<td>pDJM343</td>
<td>pUC19N carrying an ~1100 bp PCR product corresponding to a region upstream of hxuC and an ~1000 bp PCR product corresponding to a region downstream of hxuC with the kanamycin resistance marker from pKANR inserted into a unique BamHI site between the two PCR products</td>
<td>This study</td>
</tr>
<tr>
<td>pLALA29</td>
<td>pUC19N carrying an ~1100 bp PCR product corresponding to a region upstream of hxuB and an ~1000 bp PCR product corresponding to a region downstream of hxuA with the erythromycin resistance marker from pERMR inserted into a unique BamHI site between the two PCR products</td>
<td>This study</td>
</tr>
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</table>

*Sp<sup>+</sup>, spectinomycin resistance (200 μg spectinomycin ml<sup>−1</sup> in *H. influenzae*); Tc<sup>+</sup>, tetracycline resistance (3 μg tetracycline ml<sup>−1</sup> in *H. influenzae*); Kan<sup>+</sup>, kanamycin resistance (25 μg kanamycin ml<sup>−1</sup> in *H. influenzae*, 50 μg kanamycin ml<sup>−1</sup> in *E. coli*); Erm<sup>+</sup>, erythromycin resistance (6 μg erythromycin ml<sup>−1</sup> in *H. influenzae* and 200 μg erythromycin ml<sup>−1</sup> in *E. coli*); Amp<sup>+</sup>, ampicillin resistance (50 μg ampicillin ml<sup>−1</sup> in *E. coli*).

influenzae chromosomal DNA library that had been mutated using the EZ::TN < KAN-2 > Insertion kit as template (annealing at 60 °C). A product of the correct size was cloned into pCR2.1-TOPO to yield pKANR.

For deletion of hxuBA, two pairs of primers were designed to amplify the regions upstream of hxuB and downstream of hxuA respectively. The primer pair HXUBUSA and HXUBUSB amplified an ~1100 bp fragment upstream of hxuB in the PCR (annealing at 60 °C, extension time 1 min) and the primer pair HXUADSA and HXUADSB amplified an ~1000 bp fragment downstream of hxuA (annealing at 56 °C, extension time 1 min). The PCRs yielded products of the predicted size which were cloned into pCR2.1-TOPO and confirmed by DNA sequencing. Primers HXUADSA and HXUADSB added BamHI and PstI sites to the ends of one product; primers HXUBUSA and HXUBUSB added KpnI and BamHI sites to the other product. These engineered sites were subsequently utilized to subclone the PCR products into pUC19N. This strategy yielded a plasmid containing a region upstream of hxuB abutting a region downstream of hxuA with a unique BamHI site between the two fragments. The unique BamHI site was utilized to insert an ~1300 bp BamHI fragment containing the erythromycin-resistance marker from pERMR to yield pLALA29. pERMR was constructed in this laboratory and contains ermC from pE5 (Projan et al., 1987) flanked by multiple paired restriction sites (excisable by Clal, EcoRI, PstI, HindIII, KpnI, BamHI, Xmal/Smal and XbaI). The erythromycin marker was amplified from a derivative of pE5, using the primers ERM1 (5′-CTGCAGATGCATAAGCTGAATTCGAGGCGGTAC-CGGG-3′) and ERM2 (5′-GAATTCATGCATAAGCTGAATTCGAGGCCGGG-3′) (annealing at 64 °C). A product of the correct size was cloned into pCR2.1-TOPO to yield pERMR.

**Table 2. Primers**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′–3′)*</th>
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<tbody>
<tr>
<td>HXUCUSA</td>
<td>GAATTCGCGATTTTCGTCGATCAATCC</td>
</tr>
<tr>
<td>HXUCUSB</td>
<td>GAGATCCGTAATAATTAAAAAGAGG</td>
</tr>
<tr>
<td>HXUCDSC</td>
<td>GAGATCCCTACCTTTGGAAAACGCC</td>
</tr>
<tr>
<td>HXUCDSB</td>
<td>GTGCAGAGATGTTAGCAGTTACACC</td>
</tr>
<tr>
<td>HXUBUSA</td>
<td>GTGATCCGCTGCGGTGATATTATAGGC</td>
</tr>
<tr>
<td>HXUBUSB</td>
<td>GAGATCCCTATCATGCTCTATTAGTG</td>
</tr>
<tr>
<td>HXUBDSB</td>
<td>CTGACAGATTGATGATTAACGCTTTC</td>
</tr>
<tr>
<td>HXUADBSC</td>
<td>GAGATCCCTATTCATAGGAGGATG</td>
</tr>
<tr>
<td>HXUADSA</td>
<td>CTGACAGATTGATGATTAACGCTTTC</td>
</tr>
<tr>
<td>HXUADSB</td>
<td>GAGATCCCTATTCATAGGAGGATG</td>
</tr>
<tr>
<td>HXUC-A</td>
<td>CTGACAGCTTATTTGTCCATTCTTTCGAG</td>
</tr>
<tr>
<td>HXUC-B</td>
<td>CTGACAGCTTATTTGTCCATTCTTTCGAG</td>
</tr>
</tbody>
</table>

*Highlighted portions of the primer sequence (highlighted in bold) correspond to designed restriction sites added to the primer.
results (selection on 25 μg kanamycin ml⁻¹) using a modification of the static-aerobic method as previously described (Morton et al., 2004b). Antibiotic-resistant transformants were confirmed by PCR and are designated in Table 1.

**Growth studies with H. influenzae.** Growth studies were performed in hBHI supplemented as appropriate, using the Bioscreen C Microbiology Reader (Oy Growth Curves AB Ltd) as previously described (Morton et al., 2005, 2006a).

**Animals.** Specified pathogen-free (SPF), timed-pregnant Sprague–Dawley rats (Harlan) 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 biocontainment facility to prevent cross-contamination. Newborn pups from different mothers were pooled and randomly reassigned to the mothers (n=10 pups per female). Pups were weaned at 21 days. 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 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 in both 5-day-old and 30-day-old rats. The inoculum was prepared as previously described (Morton et al., 2004b). Rats were inoculated with 100 c.f.u. in 100 μl by intraperitoneal injection. To determine the actual infective dosage, aliquots of the inoculum were plated on chocolate agar containing bacitracin. At specified time intervals pups were examined for symptoms of infection, and blood specimens (50 μl) were obtained. Bacteraemia was quantified using the track-dilution procedure of Jeft et al. (1997) as previously described (Morton et al., 2004b).

**Statistics.** Statistical comparisons of growth between strains under the same growth conditions in vitro were made using the Kruskal–Wallis test. Analyses were performed using Analyse-It for Microsoft Excel v1.71 (Analyse-It Software Inc.). A P-value <0.01 was taken as statistically significant.

For in vivo studies, bacteraemic titres are expressed as mean ± S.D. typically from groups of 10 animals. Bacterial titres in blood were compared between rats challenged with different H. influenzae strains with the Kruskal–Wallis test using Analyse-It. Percentages of bacteraemic pups infected with wild-type or mutant strains were compared by the Fisher Exact Test. Analyses were performed with SigmaStat software (SPSS Inc.). A P-value <0.05 was taken as statistically significant.

**RESULTS AND DISCUSSION**

The HxuCBA proteins are highly conserved

Sequences derived from the primary translation of the cloned hxuCBA locus from strain E1a were compared with sequences available from other H. influenzae strains (identities were calculated using the Align X utility of the Vector NTI suite v.9). Sequences of one or more of the genes of the hxuCBA gene cluster are available from nine strains in addition to E1a: these strains are the type b strain DL42 (Cope et al., 1994, 1995) (GenBank accession nos U08349 and U08348), type b strain HI689 (GenBank accession no. AF536755), the capsule-deficient type d derivative Rd KW20 (Fleischmann et al., 1995) (GenBank accession no. L42023), non-typable strain N182 (Cope et al., 1994) (GenBank accession no. U08349), non-typable strain 86-028NP (Harrison et al., 2005) (GenBank accession no. CP000057), non-typable strains R2846 and R2866 (http://www.genome.washington.edu/uvwc/), non-typable strain 3224A (http://www.microgen.ouhscl.edu/) and non-typable strain HI1388 (GenBank accession no. AF536754).

Comparison of the derived protein sequences showed that HxB is the most highly conserved of the three proteins, with an overall identity between the nine available HxB sequences of 91%. HxC is only slightly less conserved than HxB, with an overall identity across the ten available sequences of 83%, while HxA is the least conserved of the three proteins, with an overall identity across the ten available sequences of 66%. In view of their high level of conservation and their localization to the outer membrane, HxC and HxB represent potential vaccine candidates for protection against a wide range of H. influenzae strains (Cripps et al., 2002; Moxon & Rappuoli, 2002).

**Growth characteristics of H. influenzae hxuC and hxuBA mutants**

The hxuCBA gene cluster is known to mediate the utilization of haem–haemopexin complexes (Cope et al., 1995). Thus we initially confirmed this phenotype for the hxuC and hxuBA mutant derivatives of strain E1a constructed here. The wild-type E1a strain grew well with haem–haemopexin as the sole haem source (Fig. 1). In contrast, neither the hxuC nor the hxuBA deletion mutant strains grew when supplied with haem–haemopexin as the sole haem source (Fig. 1). These findings confirm previously reported data for H. influenzae type b strain DL42 (Cope et al., 1995; Hanson et al., 1992). H. influenzae can utilize haem complexed either to rabbit haemopexin, as used in this study and others (Wong et al., 1994), or to human haemopexin (Stull, 1987; Pidcock et al., 1988; Cope et al., 1995; Wong et al., 1994), demonstrating that the HxuCBA system is not specific for haemopexin from humans.

Cope et al. (1995) also demonstrated that an hxuC mutant derivative of strain DL42 was unable to use low levels of free haem while mutants in either hxuB or hxuA grew well under the same conditions; the strain E1a hxuC mutant derivative was similarly unable to grow at 5 μg haem ml⁻¹ while the hxuBA grew as well as the wild-type at this haem concentration (data not shown). Since it is known that H. influenzae can use haem–albumin complexes as a haem source (Stull, 1987), we also compared the wild-type strain and its mutant derivatives for the ability to grow in this haem source. The hxuC deletion mutant derivative of E1a was unable to grow when haem was supplied as the haem–albumin complex; however, the hxuBA mutant derivative grew as well as the wild-type strain on this haem source (Fig. 1). We have also constructed hxuC and hxuBA deletion mutants in the non-typable strain HI1388 and the sequenced strain Rd KW20; the growth profiles are similar to those reported for strain E1a in growth studies with...
haem–albumin as the sole haem source (data not shown). These findings demonstrate that the haem–albumin utilization defect is mediated by the hxuC mutation rather than through polar effects on hxuB and/or hxuA, and that expression of HxuC alone, i.e. without HxUB or HxUA, is adequate for acquisition of haem from haem–albumin complexes.

Based on previous reports and data presented here, HxuC is necessary for the acquisition of haem from both haem–albumin and haem–haemopexin complexes, and for the utilization of low levels of haem in vitro (Cope et al., 1995). It has also been implicated in the acquisition of haem from haemoglobin in the absence of the Hgps (Cope et al., 2001). In view of the role of HxuC in the acquisition of haem from these various sources and the localization of this protein to the outer membrane, it is likely that HxuC represents an essential component of a common high-affinity haem acquisition pathway (Cope et al., 1995; Hanson et al., 1992; Morton & Stull, 2004). HxuC likely functions as an outer-membrane haem-binding protein and/or transmembrane transport protein. In contrast to HxuC, the HxUB and HxUA proteins are required only for utilization of haem from haem–haemopexin complexes and not from haem–albumin complexes or for the utilization of free haem. Thus, HxUA may function only as a haem–haemopexin-binding protein; HxUB is believed to function in the processing of HxUA (Cope et al., 1995). The potential importance of haem–albumin as an in vivo haem source is not addressed experimentally in this study. It remains unclear whether haem–albumin represents a significant haem source for H. influenzae in vivo. Virtually all free haem may be bound to haemopexin at non-saturating concentrations, since haemopexin has a significantly higher affinity for haem than does albumin [Kd for haemopexin 10^{-13} M versus 10^{-8} M for albumin (Koskelo & Muller-Eberhard, 1977)]. However, albumin is the major protein component of serum and occurs at concentrations of 35–50 g l^{-1} compared to a normal range of 0.5–1.5 g l^{-1} for haemopexin (Ward & Bullen, 1999; Peters, 1996; Morgan et al., 1976). Haemopexin is able to remove haem from haem–albumin complexes and effectively competes with albumin at these physiological concentrations (Morgan et al., 1976). However, at sites of localized tissue damage or erythrocyte lysis, free haem levels may saturate haemopexin and lead to haem binding by albumin; indeed, it has been proposed that released methaemoglobin can overwhelm the binding capacity of haptoglobin, haemopexin and albumin (Balla et al., 1995). In such circumstances haem–albumin may represent a significant haem source for this haem-requiring opportunistic pathogen. Additional work is necessary to clarify the potential of haem–albumin complexes to act as an in vivo haem source.

### Contribution of HxuCBA to virulence in the rat model of invasive disease

The growth-limiting effects in vitro of hxuCBA mutations on haem acquisition raised the possibility that this gene cluster is important in virulence. To determine whether proteins encoded by the hxuCBA operon contribute significantly to the virulence of H. influenzae in the infant rat model of invasive disease, a cohort of 5-day-old rat pups were challenged with either the wild-type strain E1a or the hxuC deletion mutant strain derived from E1a (HI1796). At the infective dose of 100 c.f.u., both strains were highly effective at establishing bacteraemia. All animals challenged with each strain became bacteraemic within 24 h after infection. Antibiotic-resistance profiles of recovered organisms were periodically determined to ensure that the infecting organism was the correct strain.

Infant rats challenged with the wild-type strain E1a developed noticeable tremor 2 days post-challenge but remained active and retained normal mobility and normal righting reflexes. By the third day post-infection, severe tremor indicative of meningitis was observed in all rat pups infected by the wild-type strain but not in those infected with the hxuC deletion mutant strain. The status of infant animals infected with the wild-type strain declined rapidly; they became hypothermic to touch, lost mobility and righting and died or were euthanized. A statistically significant difference in survival of infant rats after challenge with each of the strains was observed in two replicate experiments (Fig. 2a). Lethality was significantly higher by
4 days after challenge with the wild-type E1a strain compared to the hxcu mutant HI1796 (P < 0.01).

Weight gain as a function of age is a non-specific but quantitative measure of clinical wellbeing. In two replicate experiments we also observed that the genotype of the infecting strain significantly affected weight gain of infant rats following challenge. A representative experiment is shown in Fig. 2(b). Prior to challenge, the mean ± SD weights of a cohort of pups from four different dams (n = 10 per dam) did not differ from one another on days 2–5 after birth. One day post-challenge the infant animals continued to gain weight at their previous rate. However, by the second day following challenge, marked differences were noted between pups infected with the different H. influenzae strains. Mean weight gains in infants infected with the wild-type strain were arrested compared to those infected with the hxcu mutant strain HI1796. These differences were statistically significant (P < 0.002).

The differences in clinical severity caused by the mutant strain were associated with substantial reductions in their abilities to produce bacteraemia compared to the wild-type strain. The mean titre of the wild-type strain was already high (2.1 × 10^6 c.f.u. ml⁻¹) within 24 h after challenge. By 72 h it had increased to 6.9 × 10^6 c.f.u. ml⁻¹ (Fig. 2c). Most of the infant animals infected with the wild-type strain died on the third day of infection. Those that survived had uncharacteristically low bacteraemic titres. These data establish that the hxcuCBA gene cluster is important in the establishment of invasive disease in the infant rat; however, the data do not differentiate between the potential roles of HxuC and HxuB or HxuA.

We have shown differential effects on haem-source utilization of mutations in hxcu and hxbA and in addition the hxcu mutation potentially results in polar effects on hxbB and hxbA. In view of these facts, we also determined the ability of the hxbA mutant strain HI1764 to establish bacteraemia in the infant rat. Strain HI1764 was comparable to the hxcu mutant strain HI1796 in all measures used to determine virulence. One rat of ten infected with strain HI1764 died during the course of the experiment. No impact on weight gain was observed in the cohort of infant rats infected with strain HI1764, and bacteraemic titres were lower than those attained in infant rats infected with the wild-type strain by a factor comparable to that seen with the hxcu mutant HI1796 (Fig. 3). These data show that there is no difference in virulence between either an hxcuC or an hxbA mutant, while both mutants are markedly less virulent than the wild-type strain. Since in vitro the hxbA mutation affects only utilization of haem–haemopexin and not any other tested haem source, it is possible that the haem–haemopexin complex represents a significant haem source in the infant rat during bacteraemia. However, we cannot rule out the possibility that additional, as-yet-unidentified, haem sources are available in vivo and that their acquisition is affected by the hxcuCBA mutations.

Since haemoglobin–haaptoglobin and haemoglobin may represent important in vivo haem sources, and the haemoglobin–haaptoglobin binding proteins (Hgps) have been shown to be involved in the disease process in other models (Morton et al., 2004a), we additionally assessed the ability of strain E1a mutant derivatives lacking the hgp genes in combination with either the hxcu or hxbCBA genes for their ability to cause bacteraemia in the infant rat. We have shown that strain E1a possesses two hgp genes (hgpB and hgpC); as is the case with other characterized H. influenzae strains, the Hgp proteins are highly homologous to each other and are subject to phase variation via alterations of a
lacking either the hruBA genes alone (strain HI1764), the hruBA genes in combination with the hgp genes (strain HI1763) or the hruC gene in combination with the hgp genes. Based on the same criteria assessed above, there was no additive effect of mutation of the hgp genes in the presence of mutations in the hruCBA gene cluster. These findings contrast with our recent report that simultaneous mutation of the hgp genes and the hruCBA gene cluster in a second type b strain (HI689) render the mutant avirulent in the 5-day-old infant rat (Seale et al., 2006). These data indicate that strain E1a possesses either (1) alternative pathways for utilization of one or more haem sources that is not utilized by strain HI689.

Plasma haemopexin and haptoglobin levels alter significantly during mammalian development. In humans, haemopexin serum levels increase significantly between day 1 postpartum and 6 months of age; in term infants 95th percentile serum haemopexin concentrations increased from 0.44 g l$^{-1}$ at 1 day to 0.78 g l$^{-1}$ at 1 month, 0.92 g l$^{-1}$ at 2 months and reached adult levels of 1.11 g l$^{-1}$ at 6 months (Kanakoudi et al., 1995). Haptoglobin was undetectable in the serum of 60% of term infants on day 1 postpartum; by 6 months of age haptoglobin could be detected in all infants, with 95th percentile serum haptoglobin concentrations increasing from 0.44 g l$^{-1}$ at 1 month to 1.65 g l$^{-1}$ at 2 months and 3.24 g l$^{-1}$ at 6 months (Kanakoudi et al., 1995). Similar effects are seen with respect to plasma levels of haptoglobin and haemopexin in rats; in the case of haptoglobin, levels decrease immediately after birth reaching a nadir at 5 days of age and subsequently increase to attain adult levels by 30 days of age (Seale et al., 2006). Levels of haemopexin also change in the developing rat similarly to the changes seen in humans (Moldenhauer & Rose, 1970). In view of the alteration in haem-binding protein levels seen in the ageing rat, we also compared the virulence of E1a and mutant derivatives lacking hruC or hruBA in 30-day-old rats (Fig. 4). While there was a trend toward a reduced ability of the mutant strains to establish infection compared to the wild-type strain at early time points following challenge (Fig. 4a), at no time point were the changes statistically significant. This contrasts with the results in 5-day-old rats, where there was a significantly reduced ability of the mutants to establish infection compared to the wild-type strain (Fig. 2). These age-dependent differences in the ability of the hruCBA mutant strains to establish bacteraemia may reflect the age-related changes in the mammalian haem/hemoglobin binding proteins. However, despite the unaltered ability to establish bacteraemia in the 30-day-old rats, both E1a derivatives with mutations in the hruCBA gene cluster yielded lower bacteraemic titres than the wild-type strain in the 30-day-old rats (Fig. 4b).

The data presented here with respect to the impact on virulence of mutations in the hruCBA gene cluster of strain

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**Fig. 3.** Effect of an hruBA mutation alone and hruBA mutation in an hgp background and an hruC mutation in an hgp background on invasive disease in the infant rat model. Cohorts of ten 5-day-old rat pups were infected with one of three strains: the hruBA mutant strain HI1764 (○), the hruBA/hgp mutant strain HI1763 (●) or the hruC/hgp mutant strain HI1797 (△). (a) Percentage of animals surviving; (b) weights of infected rats; (c) bacteraemic titres in infected rats. Results for both weights and bacteraemic titres are expressed as means ± SD from groups of ten rats.
E1a contrast with a report on another type b strain (DL42) which stated that a mutant lacking expression of HxuA and HxuB was as virulent as the wild-type strain in the infant rat model (Hanson et al., 1992). The data in this latter study was not shown and so it is not clear what measures of virulence were determined and direct comparison with the E1a data is not possible. However, we have recently shown that an hxC mutant derivative of an additional type b strain (HI689) showed no statistically significant reduction in bacteraemic titres compared to its wild-type progenitor in 5-day-old rats (Seale et al., 2006). The effects of various mutations in the hxCBA gene cluster, examined in this and other studies, on virulence in 5- and 30-day-old rats are summarized in Table 3.

The reason for the differences between the impact on virulence of the same mutation in different strains is not clear. The hxC mutations in strains E1a and HI689 were constructed identically and there is no difference in the in vitro phenotype of the two mutants with respect to utilization of all tested haem sources. The hxCBA loci of the two strains are essentially the same and occur in identical regions of the chromosome, effectively discounting the possibility that polar effects of the mutation on flanking genes are responsible for the discrepancy. There are two major complicating factors in interpreting the differences between strains with respect to mutations in the hxCBA gene cluster: (1) the full range of available and/or utilizable haem sources in the infant rat may not be known and (2) the haem utilization pathways may differ significantly between H. influenzae strains. During invasive disease the predominant available haem sources are most likely to be haemoglobin, haemoglobin–haptoglobin complexes and

![Graph showing effect of hxC and hxB mutations on invasive disease in the weanling rat.](image)

**Fig. 4.** Effect of an hxC mutation and an hxB mutation on invasive disease in the weanling rat. Cohorts of ten 30-day-olds were infected with one of three strains: the wild-type strain E1a (●), the hxC mutant strain HI1796 (○) or the hxBA mutant strain HI1764 (△). (a) Percentage of animals with detectable bacteraemia; (b) bacteraemic titres in infected rats. Results for bacteraemic titres are expressed as means ± SD from groups of ten rats.

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*This study.
†Seale et al. (2006).
‡Hanson et al. (1992), transposon used in construction of mutant not specified, measures of virulence used not specified.
§ND, Not determined.
haem–haemopexin complexes. However, we have recently shown that myoglobin–haptoglobin complexes, which are likely to be present in low levels in blood, are a utilizable haem source for *H. influenzae in vitro* (Morton et al., 2006b), and it is possible that additional as-yet-unidentified haem sources are also present. With respect to the haem utilization pathways of different strains of *H. influenzae*, the increasing availability of complete genomic sequences of members of the species will be of significant benefit in unravelling the highly complex and redundant pathways (Morton & Stull, 2004). The genome sequence of a type b strain (strain 10810) is currently being completed (http://www.sanger.ac.uk) and the availability of both the genome sequence and the strain will help elucidate the apparently complex interplay of the various *H. influenzae* haem acquisition pathways in vivo.

**Concluding remarks**

In conclusion, we have shown that HxuC is involved in the utilization of haem complexed to albumin in *vitro*, in addition to its roles in utilization of low levels of free haem, haem complexed to haemopexin and haemoglobin. HxuC likely represents an outer-membrane haem binding/utilization protein that constitutes part of a universal high-affinity haem uptake pathway. A mutation in the *hxuB* and *hxuA* genes has no impact on utilization of haem–albumin complexes, and HxuA and HxuB appear to be involved only in haem–haemopexin utilization. This is believed to be the first report to show an impact of mutations in the HxuCBA uptake system on virulence in an animal model of human disease. Further studies are needed to clarify the interplay of the *H. influenzae* haem acquisition systems with the mammalian host in order to better understand their precise role(s) in virulence.

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