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

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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 *hxCBA* gene cluster. It was demonstrated that *hxC*, but not *hxA* or *hxB*, is also essential for the utilization of haem from haem–albumin complexes. Mutants of the type b strain E1a lacking genes in the *hxCBA* gene cluster were examined for their ability to cause bacteraemia in rat models of invasive disease. In 5-day-old rats, mutants in the *hxCBA* genes yielded a significantly reduced bacteraemic titre compared to the wild-type strain. In addition, 5-day-old rats infected with the *hxCBA* 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 (*hgps*), either alone or in combination with the *hxCBA* mutations, had no impact on virulence in 5-day-old rats. In 30-day-old rats infected with either the *hxCBA* 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 *hxCBA* mutants than in those infected with the wild-type strain. These age-related differences in the impact of mutations in the *hxCBA* gene cluster may be related to changes in levels of host haem-binding proteins during development of the rat.

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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'Brian, 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'Brian, 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

The GenBank/EMBL/DDBJ accession number for the *hxCBA* gene cluster of *H. influenzae* strain E1a is AF545479.

et al., 1999, 2004b). Utilization of the haem–haemopexin complex is mediated by the products of the *hxCBA* 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 HxC (Cope *et al.*, 1998). HxC 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 *hxA* and/or *hxB* abolish the ability to utilize the haem–haemopexin complex (Cope *et al.*, 1995). HxC is required for the utilization of both the haem–haemopexin complex and low levels of free haem (Cope *et al.*, 1995); HxC 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 HxC can be detected in the human middle ear during acute otitis media (Whitby *et al.*, 1997), and transcription of all three genes in the *hxCBA* 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 *hxCBA* 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 HxCBA proteins and to elucidate the potential role of the *hxCBA* 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-deplete growth was performed in BHI broth supplemented with only 10 µg β-NAD ml⁻¹ (haem-deplete BHI; hdBHI). *H. influenzae* were transformed 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 haemin 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 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 Operon Technologies.

Sequencing of the *hxCBA* gene cluster from *H. influenzae* strain E1a. To sequence the *hxCBA* 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. HXCUSA and HXCUSB were used to amplify an ~1100 bp fragment upstream of *hxC* (annealing temperature 56 °C, extension time 1 min); HXC-A and HXC-B to amplify an ~2500 bp fragment encompassing the entire coding sequence of *hxC* (annealing temperature 58 °C, extension time 2 min 40 s); HXCDSA and HXCDSB to amplify an ~2800 bp fragment including the entire coding sequence of *hxB* and part of *hxA* (annealing temperature 56 °C, extension time 3 min); and HXCDSA and HXCDSB to amplify an ~3800 bp fragment including the entire coding sequence of *hxA* and ~1000 bp downstream of *hxA* (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 *hxC* and *hxB* deletion mutants. Complete deletion mutants were constructed that lacked either *hxC* alone or the region encompassing *hxA* and *hxB*. These mutants were constructed as described previously (Morton *et al.*, 1999, 2004b). For deletion of *hxC*, two pairs of primers were designed to amplify regions upstream and downstream of *hxC* respectively (Table 2). Primer pair HXCUSA and HXCUSB was used in the PCR as described above and it amplified a 1073 bp product upstream of *hxC* with engineered *EcoRI* and *BamHI* sites at the ends of the amplicon to allow for directional cloning. The second primer pair, HXCDSA and HXCDSB, was designed to amplify a 966 bp product downstream of *hxC* 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 sequentially subcloned into pUC19N to yield a plasmid that contained upstream and downstream sequences of *hxC* 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 pDJM343. 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*, *Sall*, *SphI* and *HindIII*), and was constructed by performing a PCR using the primers KAN-2-1 (5'-GGATCCTGCAGTCGACGCGATGCAAGCTTCTAGATTGTGCTCAAAATCTCTGATG-3') and KAN-2-2 (5'-GAAGCTTGCA-TGCCTGCAGTTCG-3') and a random plasmid derived from a *H.*

Table 1. Strains and plasmids

Strain or plasmid	Relevant characteristic(s)*	Reference or source
<i>H. influenzae</i>		
E1a	Type b	Stull <i>et al.</i> (1984)
HI1796	E1a, $\Delta hxcC$ Kan ^r	This study
HI1764	E1a, $\Delta hxcBA$ Erm ^r	This study
HI1758	E1a, $\Delta hgpB \Delta hgpC$ Sp ^r Tc ^r	This study
HI1797	HI1758, $\Delta hxcC$ Kan ^r	This study
HI1763	HI1758, $\Delta hxcBA$ Erm ^r	This study
<i>E. coli</i>		
TOP10	Used for cloning in pCR2.1-TOPO and pUC19N	Invitrogen
Plasmids		
pCR2.1-TOPO	<i>Plac</i> , <i>lacZx</i> , Kan ^r Amp ^r , ColE1 origin, F1 origin, T7 promoter	Invitrogen
pUC19N	pUC19 with a <i>NotI</i> site added at the <i>HindIII</i> end of the polylinker	Tartof & Hobbs (1988)
pERM	pCR2.1-TOPO containing an ~1300 bp erythromycin resistance marker	This study
pKANR	pCR2.1-TOPO containing an ~1200 bp kanamycin resistance marker	This study
pDJM343	pUC19N carrying an ~1100 bp PCR product corresponding to a region upstream of <i>hxcC</i> and an ~1000 bp PCR product corresponding to a region downstream of <i>hxcC</i> with the kanamycin resistance marker from pKANR inserted into a unique <i>Bam</i> HI site between the two PCR products	This study
pLALA29	pUC19N carrying an ~1100 bp PCR product corresponding to a region upstream of <i>hxcB</i> and an ~1000 bp PCR product corresponding to a region downstream of <i>hxcA</i> with the erythromycin resistance marker from pERM inserted into a unique <i>Bam</i> HI site between the two PCR products	This study

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

Table 2. Primers

Primer	Sequence (5'–3')*
HXCUSA	GAATTC GCATTTCTGTCATCAATCC
HXCUSB	GGATCC GAATAATTAAGAGG
HXCDSA	GGATCC TTACTTTGGAAACGCCC
HXCDSB	GTGCAGG TATAGTACAGTTACACC
HXUBUSA	GGTACCT GGGGTGATTATATGCG
HXUBUSB	GGATCCC TTATGTCCTATTAATTG
HXUBDSA	GGATCCC TTTCTAAGGAGGTATGC
HXUBDSB	CTGCAGAT TGATATTAACGCTTTTGC
HXUADSA	GGATCCC TATTCAGAACACAAGG
HXUADSB	CTGCAGG TAATGCAGAAATAACTGC
HXC-A	CTGCAGC TATTTAGTCCAATTTTTCAGG
HXC-B	CTGCAGC AATAACAGAATAACGAGGTCTC

*Highlighted portions of the primer sequence (highlighted in bold) correspond to designed restriction sites added to the primer.

For deletion of *hxcBA*, two pairs of primers were designed to amplify the regions upstream of *hxcB* and downstream of *hxcA* respectively. The primer pair HXCUBUSA and HXCUBUSB amplified an ~1100 bp fragment upstream of *hxcB* 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 *hxcA* (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 *Bam*HI and *Pst*I sites to the ends of one product; primers HXCUBUSA and HXCUBUSB added *Kpn*I and *Bam*HI 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 *hxcB* abutting a region downstream of *hxcA* with a unique *Bam*HI site between the two fragments. The unique *Bam*HI site was utilized to insert an ~1300 bp *Bam*HI fragment containing the erythromycin-resistance marker from pERM to yield pLALA29. pERM was constructed in this laboratory and contains *ermC* from pE5 (Projan *et al.*, 1987) flanked by multiple paired restriction sites (excisable by *Cla*I, *Eco*RI, *Pst*I, *Hind*III, *Kpn*I, *Bam*HI, *Xma*I/*Sma*I and *Xba*I). The erythromycin marker was amplified from a derivative of pE5, using the primers ERM1 (5'-CTGCAGATCGATAAGCTTGAATTCGAGCTCGGTAC-CCGG-3') and ERM2 (5'-GAATTCATCGATCTAGAGGTACCCGG-GATCCTGCAGCATGCTATGACCATGATTACGCC-3') (annealing at 64 °C). A product of the correct size was cloned into pCR2.1-TOPO to yield pERM.

H. influenzae E1a and HI1758 were transformed to erythromycin resistance (selection on 6 µg erythromycin ml⁻¹) or kanamycin

resistance (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 hdBHI 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 Jett *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 $\pm$ SD 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 U09840 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/uwgc/>), non-typable strain 3224A (<http://www.microgen.ouhsc.edu/>) and non-typable strain HI1388 (GenBank accession no. AF536754). Comparison of the derived protein sequences showed that HxuB is the most highly conserved of the three proteins, with an overall identity between the nine available HxuB sequences of 91 %. HxuC is only slightly less conserved than HxuB, with an overall identity across the ten available sequences of 83 %, while HxuA 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, HxuC and HxuB 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

Fig. 1. Growth of the *H. influenzae* type b strain E1a, the *hxC* deletion mutant strain HI1796 and the *hxCBA* deletion mutant strain HI1764 in hdBHI supplemented with either haem-haemopexin complexes (2 µg haem equivalent ml⁻¹) or haem-albumin complexes (1 µg haem equivalent ml⁻¹). Results are for E1a in haem-albumin (◆), E1a in haem-haemopexin (◇), the *hxCBA* deletion mutant strain HI1764 in haem-albumin (■) and the *hxCBA* deletion mutant strain HI1764 in haem-haemopexin (□). The growth curves for the *hxC* deletion mutant strain HI1796 in both haem-albumin and haem-haemopexin follow exactly that for strain HI1764 in haem-haemopexin and are thus not visible on the graph. Results are means ± SD for quadruplicate results from a representative experiment.

haem-albumin as the sole haem source (data not shown). These findings demonstrate that the haem-albumin utilization defect is mediated by the *hxC* mutation rather than through polar effects on *hxB* and/or *hxA*, and that expression of HxC alone, i.e. without HxB or HxA, is adequate for acquisition of haem from haem-albumin complexes.

Based on previous reports and data presented here, HxC 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 HxC in the acquisition of haem from these various sources and the localization of this protein to the outer membrane, it is likely that HxC represents an essential component of a common high-affinity haem acquisition pathway (Cope *et al.*, 1995; Hanson *et al.*, 1992; Morton & Stull, 2004). HxC likely functions as an outer-membrane haem-binding protein and/or transmembrane transport protein. In contrast to HxC, the HxB and HxA 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, HxA may function only as a haem-haemopexin-binding protein; HxB is believed to function in the processing of HxA

(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 [K_D for haemopexin 10^{-13} M versus 10^{-8} M for albumin (Koskela & 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 HxCBA to virulence in the rat model of invasive disease

The growth-limiting effects *in vitro* of *hxCBA* mutations on haem acquisition raised the possibility that this gene cluster is important in virulence. To determine whether proteins encoded by the *hxCBA* 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 *hxC* 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 *hxC* 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

Fig. 2. Effect of an *hxC* mutation in the infant rat model of *H. influenzae* infection. Cohorts of ten 5-day-old rat pups were infected with either the wild-type strain E1a (●) or the *hxC* mutant strain HI1796 (○). (a) Percentage of animals surviving; (b) weights of infected rats; (c) bacteraemic titres in infected rats. Results for both weights and bacteraemic titres are means $\pm$ SD from groups of 10 rats. The arrow in (a) and (b) indicates the point of challenge with 100 c.f.u. of the infecting strain by intraperitoneal injection. * $P < 0.002$, ** $P < 0.01$.

4 days after challenge with the wild-type E1a strain compared to the *hxC* 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 $\pm$ 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 *hxC* 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 6.9×10^7 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 *hxCBA* 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 HxC and HxB or HxA.

We have shown differential effects on haem-source utilization of mutations in *hxC* and *hxB* and in addition the *hxC* mutation potentially results in polar effects on *hxB* and *hxA*. In view of these facts, we also determined the ability of the *hxB* mutant strain HI1764 to establish bacteraemia in the infant rat. Strain HI1764 was comparable to the *hxC* 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 *hxC* mutant HI1796 (Fig. 3). These data show that there is no difference in virulence between either an *hxC* or an *hxB* mutant, while both mutants are markedly less virulent than the wild-type strain. Since *in vitro* the *hxB* 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 *hxCBA* mutations.

Since haemoglobin–haptoglobin and haemoglobin may represent important *in vivo* haem sources, and the haemoglobin–haptoglobin 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 *hxC* or *hxB* 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

Fig. 3. Effect of an *hxCBA* mutation alone and *hxCBA* mutation in an *hgp* background and an *hxC* 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 *hxCBA* mutant strain HI1764 (○), the *hxCBA/hgp* mutant strain HI1763 (●) or the *hxC/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 $\pm$ SD from groups of ten rats.

length of CCAA nucleotide repeats within the gene (Ren *et al.*, 1999; Morton *et al.*, 1999; Cope *et al.*, 2000). Deletion of both *hgp* genes from strain E1a abolishes the ability to utilize haemoglobin-haptoglobin complexes (D. J. Morton, T. W. Seale, T. M. VanWagoner, P. W. Whitby and T. L. Stull, unpublished observation). The E1a mutant derivative lacking *hgp* genes is designated HI1758 and was unaltered in its ability to cause invasive disease in the infant rat compared to the wild-type strain (data not shown). Fig. 3 shows various measures of infection comparing the E1a mutant

lacking either the *hxCBA* genes alone (strain HI1764), the *hxCBA* genes in combination with the *hgp* genes (strain HI1763) or the *hxC* 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 *hxCBA* gene cluster. These findings contrast with our recent report that simultaneous mutation of the *hgp* genes and the *hxCBA* 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 strain HI689 is unable to use or (2) mechanisms allowing utilization of an unidentified haem source 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 concentrations increasing from 1.08 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 *hxC* or *hxCBA* 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 *hxCBA* mutant strains to establish bacteraemia may reflect the age-related changes in the mammalian haem/haemoglobin binding proteins. However, despite the unaltered ability to establish bacteraemia in the 30-day-old rats, both E1a derivatives with mutations in the *hxCBA* 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 *hxCBA* gene cluster of strain

Fig. 4. Effect of an *hxC* mutation and an *hxBA* 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 $\pm$ 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

Table 3. Effect on virulence in the rat of mutations in the *hxCBA* gene cluster compared to the wild-type strain in three strains of type b *H. influenzae*

Strain and mutation	Impact of mutations in the <i>hxCBA</i> gene cluster on virulence in:	
	5-day-old rats	30-day-old rats
E1a* ΔhxC	Decrease in bacteraemic titres Increased survival rates No effect on % bacteraemic animals	Decrease in bacteraemic titres No impact on survival rates Possible decrease in % bacteraemic animals at early time points
E1a $\Delta hxBA$	Decrease in bacteraemic titres Increased survival rates No effect on % bacteraemic animals	Decrease in bacteraemic titres No impact on survival rates Possible decrease in % bacteraemic animals
HI689† ΔhxC	No effect on bacteraemic titres No effect on % bacteraemic animals	Decrease in bacteraemic titres Decrease in % bacteraemic animals
DL42‡ transposon:: <i>hxBA</i>	No impact on virulence	ND§

*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 HxC 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. HxC likely represents an outer-membrane haem binding/utilization protein that constitutes part of a universal high-affinity haem uptake pathway. A mutation in the *hxB* and *hxA* genes has no impact on utilization of haem–albumin complexes, and HxA and HxB 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 HxCBA 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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REFERENCES

- Balla, J., Nath, K. A., Balla, G., Juckett, M. B., Jacob, H. S. & Vercellotti, G. M. (1995). Endothelial cell heme oxygenase and ferritin induction in rat lung by hemoglobin *in vivo*. *Am J Physiol* **268**, L321–L327.
- Cope, L. D., Thomas, S. E., Latimer, J. L., Slaughter, C. A., Muller-Eberhard, U. & Hansen, E. J. (1994). The 100 kDa haem:haemopexin-binding protein of *Haemophilus influenzae*: structure and localization. *Mol Microbiol* **13**, 863–873.
- Cope, L. D., Yogeve, R., Muller-Eberhard, U. & Hansen, E. J. (1995). A gene cluster involved in the utilization of both free heme and heme:hemopexin by *Haemophilus influenzae* type b. *J Bacteriol* **177**, 2644–2653.
- Cope, L. D., Thomas, S. E., Hrkal, Z. & Hansen, E. J. (1998). Binding of heme-hemopexin complexes by soluble HxA protein allows utilization of this complexed heme by *Haemophilus influenzae*. *Infect Immun* **66**, 4511–4516.
- Cope, L. D., Hrkal, Z. & Hansen, E. J. (2000). Detection of phase variation in expression of proteins involved in hemoglobin and hemoglobin-haptoglobin binding by nontypeable *Haemophilus influenzae*. *Infect Immun* **68**, 4092–4101.
- Cope, L. D., Love, R. P., Guinn, S. E., Gilep, A., Usanov, S., Estabrook, R. W., Hrkal, Z. & Hansen, E. J. (2001). Involvement of HxC outer membrane protein in utilization of hemoglobin by *Haemophilus influenzae*. *Infect Immun* **69**, 2353–2363.
- Cripps, A. W., Foxwell, R. & Kyd, J. (2002). The challenges for the development of vaccines against *Haemophilus influenzae* and *Neisseria meningitidis*. *Curr Opin Immunol* **14**, 553–557.
- Evans, R. W., Crawley, J. B., Joannou, C. L. & Sharma, N. D. (1999). Iron proteins. In *Iron and Infection: Molecular, Physiological and Clinical Aspects*, pp. 27–86. Edited by J. J. Bullen & E. Griffiths. New York, NY: Wiley.
- Fleischmann, R. D., Adams, M. D., White, O., Clayton, R. A., Kirkness, E. F., Kerlavage, A. R., Bult, C. J., Tomb, J., Dougherty, B. A. & other authors (1995). Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* **269**, 496–512.
- Genco, C. A. & Dixon, D. W. (2001). Emerging strategies in microbial haem capture. *Mol Microbiol* **39**, 1–11.
- Griffiths, E. (1999). Iron in biological systems. In *Iron and Infection: Molecular, Physiological and Clinical Aspects*, pp. 1–26. Edited by J. J. Bullen & E. Griffiths. New York, NY: Wiley.
- Gromkova, R. C., Rowji, P. B. & Koornhof, H. J. (1989). Induction of competence in nonencapsulated and encapsulated strains of *Haemophilus influenzae*. *Curr Microbiol* **19**, 241–245.
- Hanson, M. S., Pelzel, S. E., Latimer, J., Muller-Eberhard, U. & Hansen, E. J. (1992). Identification of a genetic locus of *Haemophilus influenzae* type b necessary for the binding and utilization of heme bound to human hemopexin. *Proc Natl Acad Sci U S A* **89**, 1973–1977.
- Harrison, A., Dyer, D. W., Gillaspay, A., Ray, W. C., Mungur, R., Carson, M. B., Zhong, H., Gipson, J., Gipson, M. & other authors (2005). Genomic sequence of an otitis media isolate of nontypeable *Haemophilus influenzae*: comparative study with *H. influenzae* serotype d, strain KW20. *J Bacteriol* **187**, 4627–4636.
- Jett, B. D., Hatter, K. L., Huycke, M. M. & Gilmore, M. S. (1997). Simplified agar plate method for quantifying viable bacteria. *Biotechniques* **23**, 648–650.
- Kanakoudi, F., Drossou, V., Tzimouli, V., Diamanti, E., Konstantinidis, T., Germenis, A. & Kremenopoulos, G. (1995). Serum concentrations of 10 acute-phase proteins in healthy term and preterm infants from birth to age 6 months. *Clin Chem* **41**, 605–608.
- Koskelo, P. & Muller-Eberhard, U. (1977). Interaction of porphyrins with proteins. *Semin Hematol* **14**, 221–226.
- Loeb, M. R. (1995). Ferrochelatase activity and protoporphyrin IX utilization in *Haemophilus influenzae*. *J Bacteriol* **177**, 3613–3615.
- Moldenhauer, H. & Rose, H. (1970). Entwicklung der Serumweisheit von Ratten im ersten Monat nach der Geburt. *Acta Biol Med Ger* **25**, 469–472.
- Morgan, W. T., Liem, H. H., Sutor, R. P. & Muller-Eberhard, U. (1976). Transfer of heme from heme-albumin to hemopexin. *Biochim Biophys Acta* **444**, 435–445.
- Morton, D. J. & Stull, T. L. (2004). *Haemophilus*. In *Iron Transport in Bacteria*, pp. 273–292. Edited by J. H. Crosa, A. R. Mey, & S. M. Payne. Washington, DC: American Society for Microbiology.

- Morton, D. J., Whitby, P. W., Jin, H., Ren, Z. & Stull, T. L. (1999). Effect of multiple mutations in the hemoglobin- and hemoglobin-haptoglobin-binding proteins, HgpA, HgpB, and HgpC of *Haemophilus influenzae* type b. *Infect Immun* **67**, 2729–2739.
- Morton, D. J., Bakaletz, L. O., Jurcisek, J. A., VanWagoner, T. M., Seale, T. W., Whitby, P. W. & Stull, T. L. (2004a). Reduced severity of middle ear infection caused by nontypeable *Haemophilus influenzae* lacking the hemoglobin/hemoglobin-haptoglobin binding proteins (Hgp) in a chinchilla model of otitis media. *Microb Pathog* **36**, 25–33.
- Morton, D. J., Smith, A., Madore, L. L., VanWagoner, T. M., Seale, T. W., Whitby, P. W. & Stull, T. L. (2004b). Identification of a haem utilization protein (Hup) in *Haemophilus influenzae*. *Microbiology* **150**, 3923–3933.
- Morton, D. J., Madore, L. L., Smith, A., VanWagoner, T. M., Seale, T. W., Whitby, P. W. & Stull, T. L. (2005). The heme-binding lipoprotein (HbpA) of *Haemophilus influenzae*: role in heme utilization. *FEMS Microbiol Lett* **253**, 193–199.
- Morton, D. J., VanWagoner, T. M., Seale, T. W., Whitby, P. W. & Stull, T. L. (2006a). Differential utilization by *Haemophilus influenzae* of hemoglobin complexed to the three human haptoglobin phenotypes. *FEMS Immunol Med Microbiol* **46**, 426–432.
- Morton, D. J., VanWagoner, T. M., Seale, T. W., Whitby, P. W. & Stull, T. L. (2006b). Utilization of myoglobin as a heme source by *Haemophilus influenzae* requires binding of myoglobin to haptoglobin. *FEMS Microbiol Lett* **258**, 235–240.
- Moxon, E. R. & Rappuoli, R. (2002). Bacterial pathogen genomics and vaccines. *Br Med Bull* **62**, 45–58.
- Panek, H. & O'Brian, M. R. (2002). A whole genome view of prokaryotic haem biosynthesis. *Microbiology* **148**, 2273–2282.
- Peters, T. (1996). *All About Albumin Biochemistry, Genetics, and Medical Applications*. London: Academic Press.
- Pidcock, K. A., Wooten, J. A., Daley, B. A. & Stull, T. L. (1988). Iron acquisition by *Haemophilus influenzae*. *Infect Immun* **56**, 721–725.
- Postle, K. (1990). TonB and the gram-negative dilemma. *Mol Microbiol* **4**, 2019–2025.
- Projan, S. J., Monod, M., Narayanan, C. S. & Dubnau, D. (1987). Replication properties of pIM13, a naturally occurring plasmid found in *Bacillus subtilis*, and of its close relative pE5, a plasmid native to *Staphylococcus aureus*. *J Bacteriol* **169**, 5131–5139.
- Ren, Z., Jin, H., Whitby, P. W., Morton, D. J. & Stull, T. L. (1999). Role of CCAA nucleotide repeats in regulation of hemoglobin and hemoglobin-haptoglobin binding protein genes of *Haemophilus influenzae*. *J Bacteriol* **181**, 5865–5870.
- Schlör, S., Herbert, M., Rodenburg, M., Blass, J. & Reidl, J. (2000). Characterization of ferrochelatase (*hemH*) mutations in *Haemophilus influenzae*. *Infect Immun* **68**, 3007–3009.
- Seale, T. W., Morton, D. J., Whitby, P. W., Wolf, R., Kosanke, S. D., VanWagoner, T. M. & Stull, T. L. (2006). Complex role of hemoglobin and hemoglobin-haptoglobin binding proteins in *Haemophilus influenzae* virulence in the infant rat model of invasive infection. *Infect Immun* **74**, 6213–6225.
- Smith, A. (1985). Intracellular distribution of haem after uptake by different receptors. Haem-haemopexin and haem-asialo-haemopexin. *Biochem J* **231**, 663–669.
- Smith, A. & Morgan, W. T. (1984). Hemopexin-mediated heme uptake by liver. Characterization of the interaction of heme-hemopexin with isolated rabbit liver plasma membranes. *J Biol Chem* **259**, 12049–12053.
- Smith, A. L., Smith, D. H., Averill, D. R., Marino, J. & Moxon, E. R. (1973). Production of *Haemophilus influenzae* b meningitis in infant rats by intraperitoneal inoculation. *Infect Immun* **8**, 278–290.
- Stull, T. L. (1987). Protein sources of heme for *Haemophilus influenzae*. *Infect Immun* **55**, 148–153.
- Stull, T. L., Mendelman, P. M., Haas, J. E., Schoenborn, M. A., Mack, K. D. & Smith, A. L. (1984). Characterization of *Haemophilus influenzae* type b fimbriae. *Infect Immun* **46**, 787–796.
- Tartof, K. D. & Hobbs, C. A. (1988). New cloning vectors and techniques for easy and rapid restriction mapping. *Gene* **67**, 169–182.
- Turk, D. C. (1984). The pathogenicity of *Haemophilus influenzae*. *J Med Microbiol* **18**, 1–16.
- Ward, C. G. & Bullen, J. J. (1999). Clinical and physiological aspects. In *Iron and Infection: Molecular, Physiological and Clinical Aspects*, pp. 369–450. Edited by J. J. Bullen & E. Griffiths. New York: Wiley.
- Whitby, P. W., Sim, K. E., Morton, D. J., Patel, J. A. & Stull, T. L. (1997). Transcription of genes encoding iron and heme acquisition proteins of *Haemophilus influenzae* during acute otitis media. *Infect Immun* **65**, 4696–4700.
- Whitby, P. W., VanWagoner, T. M., Seale, T. W., Morton, D. J. & Stull, T. L. (2006). Transcriptional profile of *Haemophilus influenzae*: effects of iron and heme. *J Bacteriol* **188**, 5640–5645.
- White, D. C. & Granick, S. (1963). Hemin biosynthesis in *Haemophilus*. *J Bacteriol* **85**, 842–850.
- Wong, J. C. Y., Holland, J., Parsons, T., Smith, A. & Williams, P. (1994). Identification and characterization of an iron-regulated hemopexin receptor in *Haemophilus influenzae* type b. *Infect Immun* **62**, 48–59.

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