NOTE TO USERS

Acquisition of haemoglobin-bound iron by *Histophilus somni*

by

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" There is no emotion; there is peace.
There is no ignorance; there is knowledge.
There is no passion; there is serenity.
There is no death; there is the Force."

- Jedi Code

Abstract

M.Sc. **Yannick Tremblay** **Microbiology**

Acquisition of haemoglobin-bound iron by *Histophilus somni*

Ovine (strains 9L and 3384Y) and bovine (strains 649, 2336 and 8025) isolates of *Histophilus somni* were investigated for their ability to acquire iron from haemoglobin (Hb). Bovine isolates were capable of utilizing bovine, but not ovine, porcine or human Hb as a source of iron. Ovine isolates could not obtain iron from Hb. Bovine isolates bound bovine, ovine, and human Hbs by means of the same iron-repressible receptor(s) and produced a ~120-kDa iron-repressible, outer membrane protein. Using PCR approaches, an iron-regulated operon containing *hugX* and *hugZ* homologues and a gene (*hgbA*) that encodes a TonB-dependent, Hb-binding proteins were identified in strains 649, 9L and 3384Y. In strains 9L and 3384Y, HgbA is truncated offering a possible explanation for their lack of utilization of Hb as an iron source. In strains 2336 and 8025, expression of HgbA was also subject to a form of phase variation.

Résumé

M.Sc. **Yannick Tremblay** **Microbiologie**

Acquisition du fer de l'hémoglobine par *Histophilus somni*

L'acquisition du fer de l'hémoglobine (Hb) par des isolats de boviné (souches 649, 2336 et 8025) et d'oviné (souches 9L et 3384Y) d'*Histophilus ovis* a été examinée. Les isolats de boviné pouvaient utiliser le fer de l'Hb d'origine bovine mais non de l'Hb d'origine ovine, porcine et humaine. Les isolats d'oviné ne pouvaient acquérir le fer d'aucune source d'Hb. Tous les isolats de boviné pouvaient lier l'Hb d'origine bovine, ovine et humaine par le même récepteur régularisé par le fer et produisaient une protéine de la membrane externe (~120 kDa) régularisé par le fer. En utilisant une approche favorisant le PCR, un opéron régularisé par le fer contenant des homologues à *hugX* et *hugZ* et au gène d'une protéine qui s'attache à l'Hb (HgbA) a été identifié dans les souches 649, 9L et 3384Y. Pour les souches 9L et 3384Y, HgbA est sectionnée ce qui offre une explication pour l'absence d'utilisation du fer de l'Hb par ces deux souches. Dans les souches 2336 et 8025, HgbA est aussi sujet aux variations de phase.

Contributions to knowledge

1. *H. somni* strains 649, 2336 and 8025 were shown to acquire iron from bovine Hb but not from ovine, porcine or human Hb. Strains 9L and 3384Y failed to use any of these compounds as an iron source for growth.

2. *H. somni* strains 649, 2336 and 8025 were shown bind bovine, ovine and human Hbs by means of a single, iron-repressible receptor.

3. *H. somni* strains 649, 2336 and 8025 were shown produce an iron-repressible, ~120-kDa, outer membrane protein.

4. *H. somni* was shown to possess *hugX*, *hugZ* and *hgbA* homologues that are co-transcribed and iron-regulated.

5. *hgbA* of *H. somni* strain 649 was predicted to encode an HgbA precursor that is processed to yield a mature, 124-kDa, TonB-dependent protein. In strains 9L and 3384Y, a 2-bp deletion in *hgbA* was predicted to give rise to a truncated HgbA and in strain 9L, a large insert was also shown to be present farther downstream.

6. Expression of HgbA in *H. somni* strains 2336 and 8025 was shown to be subject to a form of phase variation involving poly C tracts within the HgbA amino acid coding sequence.

List of abbreviations

EDDA	Ethylenediamine di-o-hydroxyphenylacetic acid
Hb	Haemoglobin
Hb-Hp	Haemoglobin-Haptoglobin
Hm	Haem
Hm-Hx	Haem-Haemopexin
Hp	Haptoglobin
HRP	Horseradish peroxidase
Hx	Haemopexin
Lf	Lactoferrin
ROS	Reactive oxygen species
RT-PCR	Reverse transcription-PCR
SA	Serum albumin
sTYE-H	Supplemented TYE-H
TBS	Tris-buffered saline
Tf	Transferrin
TTBS	TBS containing Tween 20
TYE-H	HEPES-buffered tryptone-yeast extract medium

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Chapter 1 - Introduction

1.1 *Histophilus somni*

The sheep pathogens, *Histophilus ovis* and *Haemophilus agni*, and the bovine pathogen, *Haemophilus somnus*, are very similar and over the years, taxonomic studies have often led to the suggestion that all three organisms belong to the same species. For example, Stephens *et al.* (1983) showed that *H. ovis*, *H. somnus* and *H. agni* are very closely related biochemically and these findings were later supported by DNA hybridization studies (Walker *et al.*, 1985; Piechulla *et al.*, 1986) and restriction endonuclease analyses (McGillivery *et al.*, 1986; Kirkham *et al.*, 1989). Interestingly, *H. ovis* and *H. somnus* can also be differentiated by restriction enzyme analyses (McGillivery *et al.*, 1986; Kirkham *et al.*, 1989) and by means of polymerase chain reaction (PCR) ribotyping (Appuhamy *et al.*, 1998). In 2003, Angen and co-workers reported the grouping of all three species based on the nucleotide sequences of 16S rRNA and the genes (*ropB*) that encode the β subunit of RNA polymerase. Based on this information, Angen *et al.* (2003) assigned all three organisms, *H. ovis*, *H. agni* and *H. somnus*, to a single species and renamed these organisms *Histophilus somni*.

H. somni is a Gram-negative, pleomorphic, capnophilic coccobacillus belonging to the family *Pasteurellaceae* in the gamma subdivision of the *Proteobacteria*. *H. somni* is a fastidious and slow-growing organism with low biochemical activity. *H. somni* is associated with a variety of conditions such as septicaemia, myocarditis, arthritis, pyaemia, mastitis, metritis, abortion, neonatal mortality, decreased fertility in ewes and rams, epididymo-orchitis, pneumonia and thrombotic meningoencephalitis (Robert, 1956; Rahaley and White, 1977; Rahaley, 1978a; Webb, 1983b; Harris and Janzen, 1989;

Philbey *et al.* 1991; Cassidy *et al.*, 1997). *H. somni* has also been isolated from the vagina and vaginal fluids of clinically normal ewes (Rahaley, 1978b; Higgins *et al.*, 1981; Harris and Janzen, 1989) and it has been suggested that the genital (Webb, 1983b) and respiratory (Philbey *et al.*, 1991) tracts are the natural habitats of *H. somni*.

1.2 Iron

Iron is the fourth most abundant element within the Earth's crust (Crichton and Pierre, 2001). Due to its limited solubility under atmospheric conditions at neutral pH, the concentration of ferric (Fe^{3+}) ions in solution is between 10^{-9} and 10^{-12} M (Braun *et al.*, 1998; Chipperfield and Ratledge, 2000). This concentration is lower than the 10^{-7} M required by most bacterial species (Griffiths, 1987). Iron is an element unconditionally required by virtually all organisms; exceptions include *Lactobacillus* species (Archibald, 1983; Weinberg, 1997; Imbert and Blondeau, 1998) and *Borrelia burgdorferi* (Possey and Gherardini, 2000). The range of redox potentials (~ –300 to +700 mV) of the two predominant states of oxidation of iron, ferrous (Fe^{2+}) and ferric (Fe^{3+}) (Braun *et al.*, 1998; Aisen *et al.*, 2001), is used by organisms to their benefit with iron being used as a cofactor in many enzymes and as a component of the respiratory chain (Crichton and Pierre, 2001).

Although iron is required by virtually all organisms, it can also be toxic. Iron must be sequestered to minimize its interaction with reactive oxygen species (ROS) (Andrews *et al.*, 2003). ROS are natural by-products of oxygen metabolism and include partially reduced derivatives of molecular oxygen (Fridovich, 1995). Although the major ROS, superoxide anion (O_2^-) and hydrogen peroxide, are mildly reactive, interaction between

iron and ROS, as described by the Haber-Weiss reaction (see Fig. 1.1), results in the formation of highly reactive and damaging hydroxyl radicals. Briefly, ferric complexes are reduced by superoxide to ferrous complexes and the resulting ferrous complexes can then interact with H_2O_2 in what is known as the Fenton reaction. It is hypothesised that iron reduction occurs sporadically due to the low concentration ($\sim 10^{-10}$ M) of superoxide (Pierre and Fontecave, 1999; Andrews *et al.*, 2003). However, superoxide concentrations might be sufficient to cause the release of iron from the exposed [4Fe-4S] clusters of dehydratase-lyase family members (Andrews *et al.*, 2003).

Bacterial cells are protected against oxidative damage by several antioxidant enzymes, such as superoxide dismutase, and by DNA repair mechanisms. Elevation in the intracellular concentrations of superoxide and hydroperoxide appear to initiate the induction of the antioxidant enzyme response (Storz and Imlay, 1999). Pierre and Fontecave (1999) also suggested that the ratio of $[O_2]$ to $[O_2^-]$ acts as a natural protection against oxidation damage.

1.3 Iron acquisition by bacteria

1.3.1 Iron sources within a host

The concentration of free ferric ions in solution in the presence of oxygen and at neutral pH is below that required by most bacteria. To add to the challenge of acquiring iron, pathogens of vertebrates encounter an extremely iron-restricted environment *in vivo*; the concentration of free iron in the extracellular fluids of a host is estimated to be 10^{-18} M (Griffiths, 1987). The free iron in vertebrates is sequestered by carrier and storage proteins such as transferrin (Tf), lactoferrin (Lf), haem (Hm), haemoglobin (Hb) and

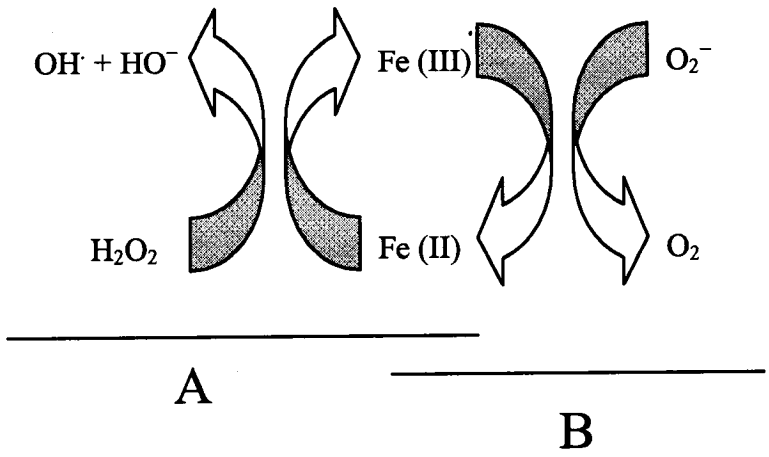


Fig. 1.1. The Haber-Weiss cycle. The Fenton reaction and iron reduction steps are represented on the left (A) and right (B), respectively.

ferritin (Griffiths, 1987; Mietzner and Morse, 1994). The concentration of sequestered iron is more than sufficient to meet the needs of the bacterial pathogen; however, pathogens must be able to release the iron. Most vertebrate iron is intracellular and associated with Hm, Hb and ferritin. To gain access to these iron supplies, pathogens must first inflict damage to the host's tissues (Mietzner and Morse, 1994). Although iron-carrier proteins and storage proteins are then released, the bacterial pathogen must overcome other host defences. Free Hm is bound rapidly by haemopexin (Hx) and serum albumin (SA) and free Hb dissociates into its dimeric form and/or is bound by haptoglobin (Hp) and these complexes are removed by the liver (Mietzner and Morse, 1994; Wandersman and Stojiljkovic, 2000). Tf and Lf are iron transport proteins present in the serum and mucosal secretions, respectively (Griffiths, 1987; Aisen, 1998). These sources of iron can be easily accessed by invading bacteria. Although less than 40% of the Tf and Lf molecules are iron-loaded, the iron held by these proteins is sufficient to support microbial growth. Pathogenic and commensal bacteria can also obtain iron from iron-chelating compounds, known as siderophores, produced by other mircoorganisms (Andrews *et al.*, 2003). For example, *Escherichia coli* can obtain iron from the fungal siderophore, ferrichrome (Braun *et al.*, 1998). Although pathogens can grow *in vivo*, the host iron-binding proteins will limit the growth rate within the host. Therefore, iron availability will have an impact on the invasion process.

1.3.2 Siderophore-dependent iron acquisition

Siderophores (derived from Greek and meaning iron carrier) are low molecular mass (< 1000 Da), non-protein molecules with high specificity and affinity ($K_{aff} > 10^{30}$) for ferric ions (Griffiths, 1987; Mietzner and Morse, 1994; Braun *et al.*, 1998; Byers and

Arceneaux, 1998; Ratledge and Dover, 2000; Andrews *et al.*, 2003). More than 500 siderophores have been identified and known to be synthesized by bacteria, fungi, yeast and monocotyledonous plants (Ratledge and Dover, 2000; Andrews *et al.*, 2003). The majority of siderophores are water-soluble and are excreted by cells, however, some are located within the membrane (De Voss *et al.*, 1999; Ratledge and Dover, 2000) and some are lipophilic (Martinez *et al.*, 2000). The majority of the iron-binding ligands of siderophores fall within three distinct chemical groups, hydroxamate, α-hydroxycarboxylate and catechol, and these ligands form hexadentate octahedral complexes with ferric iron (Winkelmann, 2002). The affinity of siderophores for iron is high enough to remove iron from ferritin, Tf and Lf but not from Hm (Ratledge and Dover, 2000; Winkelmann, 2002).

Siderophore synthesis is largely independent of primary metabolism and depends on common precursors such as citrate, amino acids, dihydroxybenzoates and N^5-acyl-N^5-hydroxyornithine (Winkelmann, 2002). Peptides attached to siderophores are assembled by non-ribosomal peptide synthetases. Siderophores tend to be cyclic due to the advantages offered by cyclization such as complex stability, chemical stability and protection against degradative enzymes (Winkelmann, 2002). Several gene products are usually responsible for the biosynthesis of siderophores and the genes tend to be organized in operons (Ratledge and Dover, 2000). Once synthesized, siderophores are likely to be exported from the cell by specific transport proteins (Andrews *et al.*, 2003), as in *E. coli* (Furrer *et al.*, 2002).

Siderophores containing ferric ions are too large (> 600 Da) to be transported across the outer membrane by porins (Andrews *et al.*, 2003). Therefore, highly-ligand-

specific membrane receptors are required for internalization of siderophores (Winkelmann, 2002). In Gram-negative organisms, the transport of siderophores is by means of TonB-dependent outer membrane receptors. Analysis of the crystal structures of three of these receptors, FepA (Buchanan *et al.*, 1999), FecA (Ferguson *et al.*, 2002) and FhuA (Ferguson *et al.*, 1998), revealed that each of these receptors consists of a 22-β-stranded barrel that forms a channel that spans the outer membrane and an N-terminal "plug" that blocks the periplasmid end (Andrews *et al.*, 2003). Once the ferri-siderophore complex is bound to a receptor, conformational changes in the exterior loop and periplasmic channel occur and it is thought that these conformational changes allow for the transport of the ferri-siderophore into the periplasm (Andrews *et al.*, 2003). Once in the periplasm, the siderophore is bound by a siderophore-binding protein (e.g., FhuD) and presented to an ABC module consisting of a permease (e.g., FhuB) located in the cytoplasmic membrane and an energy-delivering ATPase (e.g., FhuC) (Braun *et al.*, 1998; Braun and Killmann, 1999; Andrews *et al.*, 2003). In the cytoplasm, ferrireductase reduces Fe^{3+} to Fe^{2+} (Ratledge and Dover, 2000). Since siderophores have a much lower affinity for Fe^{2+} than for Fe^{3+}, reduction of the iron promotes its removal from the siderophore (Neilands, 1995; Ratledge and Dover, 2000). Once the iron is removed, the siderophore is either recycled or degraded by an esterase (Winkelmann, 2002). In the case of enterochelin, hydrolysis of the molecule by an esterase is required prior to reductive release (Griffiths, 1987; Ratledge and Dover, 2000).

The expression of siderophore-biosynthetic genes is regulated by the ferric uptake regulator (Fur); however, some systems are regulated in a more complex fashion. Since some organisms can produce uptake systems for exogenous siderophores, regulation by

Fur alone would permit the wasteful synthesis of uptake systems in the absence of the appropriate siderophore. For example, in *E. coli*, the ferric citrate uptake system is expressed only under iron-restricted conditions in the presence of ferric citrate (Braun, 1997). Binding of ferric citrate to the ferric citrate receptor (FecA) initiates a signal transduction cascade involving FecA, the cytoplasmic regulatory protein, FecI, and the cytoplasmic membrane protein, FecR. It is believed that FecR transfers the signal from FecA to FecI, activating FecI (Welz and Braun, 1998; Enz *et al.*, 2000). This process induces the transcription of the *fecABCDE* genes (see e.g. Braun, 1997; Angerer and Braun, 1998; Welz and Braun, 1998; Enz *et al.*, 2000). The regulatory genes, *fecIR*, are regulated by Fur (Angerer and Braun, 1998).

1.3.3 Siderophore-independent receptor-mediated iron acquisition

Siderophore-independent receptor-mediated mechanisms for iron acquisition have been identified in both Gram-negative and Gram-positive bacteria. Due to the overwhelming amount of information available, and space limitations, the discussion of such mechanisms will concentrate on Gram-negative organisms, most specifically, members of the families *Neisseriaceae* and *Pasteurellaceae*.

1.3.3.1 Tf- and Lf-binding proteins

Neisseriaceae and *Pasteurellaceae* do not produce siderophores but are capable of acquiring iron from Tf and Lf in a contact-dependent manner (Gray-Owen and Schryvers, 1996). This was first demonstrated in the *Neisseriaceae* by Archibald and DeVoe (1979); Tf in a dialysis bag could not serve as a source of iron for *Neisseria meningitidis*, whereas

free Tf could. Mickelson *et al.* (1982) demonstrated acquisition of Lf-bound iron by *N. meningitidis*. Subsequently, similar siderophore-independent mechanisms for the acquisition of Tf- and Lf-bound iron were identified in *Neisseria gonorrhoeae* (McKenna *et al.*, 1988; West and Sparling, 1985), *Moraxella catarrhalis* (Campagnari *et al.*, 1994) and *Moraxella bovis* (Bonnah *et al.*, 1995). Unlike the pathogenic neisseriae, the *Pasteurellaceae* cannot acquire iron from Lf. Receptor-mediated iron acquisition from Tf has been demonstrated to occur in several pathogenic members of the *Pasteurellaceae* including *Haemophilus influenzae* (Herrington and Sparling, 1985; Pidcock *et al.*, 1988), *Haemophilus parasuis* (Charland *et al.*, 1995), *Actinobacillus pleuropneumoniae* (Gonzalez *et al.*, 1990; D'Silva *et al.*, 1995), *Actinobacillus suis* (Bahrami *et al.*, 2003), *Mannheimia haemolytica* (Ogunnariwo and Schryvers, 1990; Yu *et al.*, 1992), *Pasteurella multocida* (Ogunnariwo *et al.*, 1991; Veken *et al.*, 1996) and *H. somni* (Ogunnariwo *et al.*, 1990; Yu *et al.*, 1992; Yu and Schryvers, 1994; Ekins and Niven, 2001; Ekins *et al.*, 2004a).

Acquisition of Tf- and Lf-bound iron is discriminatory in that pathogens can acquire iron only from host Tf or Lf (see e.g., Niven *et al.*, 1989; Ogunnariwo *et al.*, 1990; Schryvers and Gonzalez, 1990; Bonnah *et al.*, 1995; Charland *et al.*, 1995). While the pathogenic neisseriae and most members of the *Pasteurellaceae* acquire iron from a single species of Tf, *M. haemolytica* (Yu *et al.*, 1992), *P. multocida* (Ogunnariwo and Schryvers, 2001) and *H. somni* (Yu and Schryvers, 1994; Ekins and Niven, 2001; Ekins *et al.*, 2004a) are somewhat different in that these organisms can bind, and acquire iron from, at least three different ruminant Tfs: bovine, ovine and caprine.

The receptors responsible for the binding of Tf and Lf usually consist of two outer-membrane proteins. In such bipartite receptors, the larger components are named

transferrin-binding protein A (TbpA) and lactoferrin-binding protein A (LbpA). TbpA and LbpA are integral, TonB-dependent outer-membrane receptors and are structurally similar to siderophore receptors. The major difference is that TbpA and LbpA are ~20 kDa larger than siderophore receptors and therefore, TbpA and LbpA have larger surface loops (Gray-Owen and Schryvers, 1996; Schryvers and Stojiljkovic, 1999). Unlike siderophore receptors, which allow transport of siderophores into the periplasm, iron is removed from Tf or Lf by TbpA or LbpA, respectively, processes that require energy (Cornelissen *et al.*, 1997; McKenna *et al.*, 1988). TbpB and LbpB refer to the smaller components of the Tf and Lf receptors, respectively. TbpB and LbpB are surface-exposed lipoproteins and, presumably, are anchored to the outer membrane (Gray-Owen and Schryvers, 1996). The exact roles of TbpB and LbpB are unknown. It has been shown that TbpB improves the efficiency of iron acquisition from Tf (Anderson *et al.*, 1994; Cornelissen *et al.*, 1994; Gray-Owen *et al.*, 1995; Luke and Campagnari, 1999); however, it does not facilitate the release of the metal from the Tf (Nemish *et al.*, 2003).

In contrast to the situation in the pathogenic neisseriae and many members of the *Pasteurellaceae*, *P. multocida* (Ogunnariwo and Schryvers, 2001) and *H. somni* (Ekins and Niven, 2001; Ekins and Niven, 2002; Ekins *et al.*, 2004a) produce single-component Tf receptors. These proteins are homologues of TbpA and are currently referred to as TbpA2 (Ekins *et al.*, 2004a). Interestingly, a bovine isolate of *H. somni* has also been shown to possess two systems for the acquisition of Tf-bound iron, a bipartite system specific for bovine Tf (TbpA/TbpB) and a single-component system (TbpA2) that recognizes ruminant Tfs (Ekins *et al.*, 2004a).

Once iron is in the periplasm, it is transported by the iron-repressible *fbpABC* gene products (Adhikari *et al.*, 1995, 1996; Khun *et al.*, 1998; Kirby *et al.*, 1998; Khun *et al.*,

2000; Ekins *et al.*, 2004b). The FbpABC transport system is similar to those used for the transport of siderophores; FbpA binds and then transports iron to the permease, FbpB, and FbpC supplies the energy for the translocation of iron into the cytoplasm (Adhikari *et al.*, 1996).

In virtually all species investigated, the genes encoding TbpA, TbpB, LbpA and LbpB are organised in a similar manner: *tbpB* precedes *tbpA* and *lbpB* precedes *lbpA* (Legrain *et al.*, 1993; Anderson *et al.*, 1994; Gonzalez *et al.*, 1995; Gray-Owen *et al.*, 1995; Ogunnariwo *et al.*, 1997; Bonnah and Schryvers, 1998; Du *et al.*, 1998; Lewis *et al.*, 1998a; Petterson *et al.*, 1998; Biswas *et al.*, 1999; Ekins *et al.*, 2004a). In *M. catarrhalis*, however, *tbpB* is downstream of *tbpA* and *tbpA* and *tbpB* are separated by an unknown open reading frame (Myers *et al.*, 1998). The genetic organization of the Tf receptor genes and the identification of regulatory elements, including Fur boxes, upstream of only *tbpB* suggest that *tbpA* and *tbpB* are arranged as an operon (*tbpBA*) (Legrain *et al.*, 1993; Anderson *et al.*, 1994; Gonzalez *et al.*, 1995; Gray-Owen *et al.*, 1995; Ogunnariwo *et al.*, 1997; Bahrami *et al.*, 2003; Ekins *et al.*, 2004a). Experimental evidence for the production of a polycistronic *tbpBA* message has been obtained with *N. gonorrhoeae* (Anderson *et al.*, 1994; Ronpirin *et al.*, 2001) and *A. suis* (Bahrami *et al.*, 2003). Although the arrangement of *lbpA* and *lbpB* is similar to that of *tbpA* and *tbpB*, their coding regions are different in that *lbpB* and *lbpA* overlap in the pathogenic neisseriae (Lewis *et al.*, 1998a; Biswas *et al.*, 1999); in *M. catarrhalis*, *lbpB* and *lbpA* are separated by 184 bp (Du *et al.*, 1998). Based on the iron-repressible nature of the Tbps and Lbps, the identification of *fur* homologues in several members of the *Neisseriaceae* and *Pasteurellaceae* and the identification of Fur boxes within the *tbp* and *lbp* promoter regions, the expression of the Tf- and Lf-binding-protein genes is believed to be regulated

by Fur (Anderson *et al.*, 1994; Petterson *et al.*, 1994; Gonzalez *et al.*, 1995; Gray-Owen *et al.*, 1995; Ogunnariwo *et al.*, 1997; Du *et al.*, 1998; Petterson *et al.*, 1998; Ekins and Niven, 2002; Bahrami *et al.*, 2003; Ekins *et al.*, 2004a). Notably, in *H. somni*, TbpA2 and TbpB are also subject to phase variation (Ekins and Niven, 2003; Ekins *et al.*, 2004a).

1.3.3.2 Hm- and Hb-binding proteins

Siderophores are not capable of sequestering iron from Hm (Winkelmann, 2002). Several bacteria, however, are known to use receptor-mediated mechanisms to obtain iron from Hm and a variety of haemoproteins. In Gram-negative bacteria, these mechanisms involve distinct components that can be divided into three subfamilies: the enterobacterial Hm/Hb receptor, haemophores, and the *Vibrio*, *Neisseriaceae* and *Pasteurellaceae* Hm/Hb receptor (Perkins-Balding *et al.*, 2003). The present discussion will focus on the latter two subfamilies since these contain the components that are produced by members of the *Pasteurellaceae* (and *Neisseriaceae*).

1.3.3.2.1 Haemophores

Haemophores have been identified, to date, in *Serratia marcescens* (Létoffé *et al.*, 1994), *Pseudomonas aeruginosa* (Létoffé *et al.*, 1998; Ochsner *et al.*, 2000), *Yersinia pestis* (Rossi *et al.*, 2001) and *H. influenzae* (Cope *et al.*, 1994). Haemophores are secreted Hm-sequestering proteins that bind Hm, Hb and Hx in the extracellular fluids of the host and transport Hm back to the bacteria cells. Like siderophores, haemophores require a TonB-dependent outer membrane receptor and secretion proteins, and are regulated by Fur. The acquisition systems found in the *Enterobacteriaceae* appear to be unrelated to the one found in *H. influenzae*. The *H. influenzae* haemophore, HxuA (~100

kDa), is much larger than the enterobacterial HasA (~20 kDa). HxuA has been found to be associated with the membrane and in the culture supernatant (Cope *et al.*, 1994) whereas HasA is found only in the culture supernatant (Létoffé *et al.*, 1994, 1998; Ochsner *et al.*, 2000; Rossi *et al.*, 2001). The receptor for HxuA has not yet to been identified; however, a gene, *hxuC*, upstream of *hxuA* has been proposed to encode the receptor. HxuC has been demonstrated to be an outer membrane protein (Cope *et al.*, 1995) and to play a role in acquisition of iron from Hb (Cope *et al.*, 2001), haem-haemopexin (Hm-Hx) complexes and Hm, at low concentrations (Cope *et al.*, 1995). HxuA, however, is associated only with acquisition of iron from Hm-Hx complexes (Hanson *et al.*, 1992a). Acquisition of Hb-bound iron could not be abolished in *hxuA* or *hxuC* knock-out mutants due to the presence of three other Hb-binding proteins (Morton *et al.*, 1999). Enterobacterial haemophores have been associated with the acquisition of iron from Hm and Hb (Létoffé *et al.*, 1994, 1998; Ochsner *et al.*, 2000; Rossi *et al.*, 2001). The haemophores are secreted by membrane proteins. In *Enterobacteriaceae*, two proteins, HasD and HasE, provide this function and are encoded in an operon with *hasR* and *hasA* (Létoffé *et al.*, 1998, 1999; Ochsner *et al.*, 2000; Rossi *et al.*, 2001). In *Y. pestis* and *S. marcescens*, *hasB* codes for a TonB-like protein and is also part of the *hasRADE* operon (Paquelin *et al.*, 2001; Rossi *et al.*, 2001). In *H. influenzae*, only one membrane protein, HxuB, is responsible for secretion of HxuA and *hxuB* is present in an operon that includes *hxuA* but not *hxuC* (Hanson *et al.*, 1992a; Cope *et al.*, 1994).

1.3.3.2.2 Hm and Hb receptors of *Neisseriaceae* and *Pasteurellaceae*

Several members of the *Neisseriaceae* and *Pasteurellaceae* have been shown to be capable of binding, and/or obtaining iron from, Hm (Dyer *et al.*, 1987; Stull, 1987; Williams *et al.*, 1990; Lee, 1992). *H. influenzae*, *Haemophilus parainfluenzae* and *Haemophilus paraphrophilus* can also acquire iron from Hm bound to Hx and SA (Williams *et al.*, 1990) whereas pathogenic neisseriae can not (Dyer *et al.*, 1987). Intact cells of *H. influenzae* can also bind Hx and a 57-kDa outer membrane protein has been identified as the receptor (Wong *et al.*, 1994). Several other Hm-binding proteins of *H. influenzae* have been affinity-isolated (Hanson and Hansen, 1991; Hanson *et al.*, 1992b; Lee, 1992; Wong *et al.*, 1994, 1995) and some of these proteins appear to be conserved among certain *Haemophilus* species (Hanson *et al.*, 1992b; Wong *et al.*, 1995). Hm-binding proteins have also been identified in *Haemophilus ducreyi* (Thomas *et al.*, 1998), *N. meningitidis* (Lee, and Hill, 1992; Lee, 1994), *P. multocida* (Garrido *et al.*, 2003; Bosch *et al.*, 2004) and *A. pleuropneumoniae* (Archambault *et al.*, 2003).

Binding and/or utilization of Hb by intact cells and/or membranes has also been observed in the pathogenic neisseriae (Dyer *et al.*, 1987; Lee and Hill, 1992; Lewis and Dyer, 1995; Stojiljkovic *et al.*, 1995, 1996; Lewis *et al.*, 1997), *Haemophilus* species (Stull, 1987; Williams *et al.*, 1990, Frangipane *et al.*, 1994; Elkins, 1995; Elkins *et al.*, 1995; Ren *et al.*, 1998; Jin *et al.*, 1999; Morton *et al.*, 1999; Cope *et al.*, 2000), *P. multocida* (Bosch *et al.*, 2002; Cox *et al.*, 2003), *A. pleuropneumoniae* (Archambault *et al.*, 1999, 2003; Srikumar *et al.*, 2004) and *Actinobacillus actinomycetemcomitans* (Hayashida *et al.*, 2002) and the genes encoding the TonB-dependent Hb-binding proteins responsible for this binding and utilization have been identified. Interestingly, some members of the *Neisseriaceae* and *Pasteurellaceae*, including *H. influenzae* (Morton *et*

al., 1999; Cope *et al.*, 2000), some strains of *N. meningitidis* (Lewis and Dyer, 1995; Stojiljkovic *et al.*, 1996; Lewis *et al.*, 1999; Perkins-Balding *et al.*, 2004) and *P. multocida* (Bosch *et al.*, 2002; Cox *et al.*, 2003; Bosch *et al.*, 2004), possess more than one Hb-binding protein. Hb-Hp complexes may also serve as iron sources for *H. influenzae* (Stull, 1987; Maciver *et al.*, 1996), *N. meningitidis* and *N. gonorrhoeae* (Dyer *et al.*, 1987; Lewis and Dyer, 1995). While *H. influenzae* type b (Morton *et al.*, 1999), *N. meningitidis* and *N. gonorrhoeae* (Lewis and Dyer, 1995; Perkins-Balding *et al.*, 2004) produce receptors that recognize both Hb and Hb-Hp complexes, nontypeable *H. influenzae* possesses two types of Hb-binding protein, one that binds Hb-Hp complexes specifically (Maciver *et al.*, 1996) and one that recognizes Hb as well as Hb bound to Hp (Cope *et al.*, 2000).

The Hb- and Hb-Hp-binding proteins (HpuA/HpuB) of the pathogenic neisseriae are unique in that they are the only two-component Hb receptor systems so far identified (Lewis *et al.*, 1997). As with *tbpBA*, the *hpuA* gene, which encodes the smaller component (HpuA) is upstream of the *hpuB* gene, which encodes the larger (HpuB), and both genes (*hpuAB*) are cotranscribed to yield a bicistronic mRNA (Lewis *et al.*, 1997). Unlike the situation with TbpB, HpuA is required for the utilization of iron from Hb (Chen *et al.*, 2002). However, a point mutation near the 3'-end of *hpuB* allowed a HpuA mutant to utilize Hb and it was thus proposed that HpuA is responsible for the tight binding of Hb (Chen *et al.*, 2002). The gene that encodes the Hb-binding protein, HgbA, of *P. multocida* is also located in an operon along with two smaller open reading frames, PM0298 and PM0299, that are homologues of *Plesiomonas shigelloides hugX* and *hugZ* (Bosch *et al.*, 2002). HugX and HugZ are believed to play an important role in the

acquisition of Hm-bound iron but their exact contributions to this process remain unknown (Henderson *et al.*, 2001; Bosch *et al.*, 2002).

Unlike the Tf- and Lf-binding proteins, almost none of the Hb receptors are species specific (Frangipane *et al.*, 1994; Elkins, 1995; Stojiljkovic *et al.*, 1996). The only exception is HmbR of the pathogenic neisseriae, which appears to bind human Hb preferentially (Stojiljkovic *et al.*, 1996). The fact that Hb receptors are usually non-specific with respect to host Hb suggests that Hb is bound via the Hm moiety (Elkins, 1995). This is reinforced by the findings that some Hb-binding proteins do not differentiate between Hb and Hm (Lee, 1992; Archambault *et al.*, 2003; Bosch *et al.*, 2004) and between Hb, Hp and Hb-Hp complexes (Lewis and Dyer, 1995; Jin *et al.*, 1999). However, some Hm-, Hb-Hp-, Hx- and Hb-binding proteins bind the specified substrates only (Wong *et al.*, 1994; Maciver *et al.*, 1996; Bosch *et al.*, 2004), suggesting that several different mechanisms might be involved in the binding of Hm, haemoproteins and haemoprotein complexes.

1.3.3.2.3 Regulation of expression of Hm and Hb receptors of *Neisseriaceae* and *Pasteurellaceae*

Hm- and Hb-binding proteins are iron-repressible in *H. influenzae* (Lee, 1992; Wong *et al.*, 1994; Maciver *et al.*, 1996; Hasan *et al.*, 1997), *H. ducreyi* (Elkins *et al.*, 1995), neisseriae (Lee and Hill, 1992; Lee, 1994; Lewis and Dyer, 1995; Stojiljkovic *et al.*, 1996; Lewis *et al.*, 1997), *P. multocida* (Bosch *et al.*, 2002; Cox *et al.*, 2003), and *A. pleuropneumoniae* (Archambault *et al.*, 2003; Srikumar *et al.*, 2004). Fur boxes have also been identified upstream of most of the sequenced gene encoding Hm- and Hb-binding-proteins suggesting that Fur might play a role in the regulation of expression (Elkins *et*

al., 1995; Stojiljkovic *et al.*, 1995; Lewis *et al.*, 1997; Ren *et al.*, 1998; Cope *et al.*, 2000; Cox *et al.*, 2003; Srikumar *et al.*, 2004). In *H. influenzae* and *H. ducreyi*, Hm, in addition to iron, would also appear to be involved in the regulation of expression of Hm- and Hb-binding proteins (Hanson *et al.*, 1992b; Elkins, 1995; Thomas *et al.*, 1998). Hasan *et al.* (1997) demonstrated that in *H. influenzae*, protoporphyrin IX also down-regulates the expression of Tf, Hx and Hb receptors. Regulation of the *P. multocida hbpA* gene, which encodes a Hm-binding protein, is also unusual; although *hbpA* is iron-regulated, it is Fur-independent by FURTA assay and the promoter region upstream of *hbpA* lacks a typical Fur box (Garrido *et al.*, 2003). Interestingly, HbpA, and perhaps other Hm- and Hb-binding proteins, of *P. multocida* is also subject to phase variation with a polyadenosine tract in *hbpA* being responsible for the expression of two functional Hm-binding polypeptides (Garrido *et al.*, 2003; Bosch *et al.*, 2004). Phase variation of Hb-binding proteins has also been observed in *H. influenzae* (Ren *et al.*, 1998, 1999; Jin *et al.*, 1999; Morton *et al.*, 1999; Cope *et al.*, 2000), *H. ducreyi* (Stevens *et al.*, 1996) and pathogenic neisseriae (Stojiljkovic *et al.*, 1996; Chen *et al.*, 1998; Lewis *et al.*, 1999; Richardson and Stojiljkovic, 1999). In *H. influenzae* and the neisseriae, phase variation involves CCAA repeats and polyguanine tracts in the structural genes, respectively (Ren *et al.*, 1999; Richardson and Stojiljkovic, 1999).

1.3.3.2.4 Hm binding and internalization

The mechanisms involved in the internalization of Hm and/or iron are not well understood. The best characterized system for Hb binding is the HmbR system of *N. meningitidis* (Perkins-Balding *et al.*, 2003). Two putative extracellular loops (L2 and L3) near the middle of the protein are required for binding of Hb. In the middle of the

carboxyl end, three extracellular loops, L6, L7, which contains highly conserved protein motifs (FRAP and NPNL), and L10 are important for utilization of Hb but not for its binding. Data collected using mutants indicate that a similar situation is the case in *A. pleuropneumoniae* (Srikumar *et al.*, 2004). In *A. pleuropneumoniae* (Bélanger *et al.*, 1995; Archambault *et al.*, 1999) and *A. actinomycetemcomitans* (Grenier *et al.*, 1997), the binding of Hb to its receptor might also be facilitated by lipopolysaccharides (LPS). Once Hb binds to its receptor, Hm is removed and transported to the periplasm in a TonB-dependent manner (Stojiljkovic and Srinivasan, 1997; Elkins *et al.*, 1998; Lewis *et al.*, 1998b). In the periplasm, Hm is not transported by an Fbp system (Khun *et al.*, 1998) but is most likely transported to the cytoplasm by HemTUV-like proteins. The HemTUV protein system is an ABC transporter system that has been characterized in *Yersinia enterocolitica*, and this system is promoted as a model for Hm-specific ABC transporters in Gram-negative bacteria (Perkins-Balding *et al.*, 2004). Once in the cytoplasm, Hm is probably degraded by a Hm oxygenase. A Hm oxygenase in *N. meningitidis*, HemO, has been shown to be required for the utilization of Hm and haemoproteins as sources of iron (Zhu *et al.*, 2000).

1.3.3.2.5 Role of Hm- and Hb-binding proteins in virulence and Hm- and Hb-binding proteins as potential vaccine components

Hb-binding-protein genes are expressed during infection (Whitby *et al.*, 1997; Bosch *et al.*, 2003) indicating that Hb receptors may be involved in the acquisition of iron during an infection (Jerse *et al.*, 2002). As shown in infection models, mutants of *H. influenzae* (Morton *et al.*, 2004) and *H. ducreyi* (Stevens *et al.*, 1996; Al-Tawfiq *et al.*, 2000) that lack all Hb-binding proteins exhibit decreased virulence. It has been suggested

that the Hb-binding proteins of *H. influenzae* and *H. ducreyi* might play a role in the late stages of infection and for bacterial proliferation. Mutants of *N. meningitidis* that could not use Hb as an iron source were reduced significantly in numbers during infection but retained the ability to cross the blood brain barrier (Stojiljkovic *et al.*, 1995). In *P. multocida*, a mutation in *hgbA* or *hgbB* did not affect virulence (Bosch *et al.*, 2002; Cox *et al.*, 2003); however, since *P. multocida* may possess both HgbA and HgbB, and perhaps other Hb-binding proteins, the contributions of HgbA and HgbB to virulence remain to be determined (Bosch *et al.*, 2002; Cox *et al.*, 2003; Bosch *et al.*, 2004). The presence of several Hb-binding proteins could potentially support the multiplication of cells when one of the receptors is under immunological pressure or inactivated by mutation (Bosch *et al.*, 2002).

Hm- and Hb-binding proteins have the potential to be good vaccine components in that some of these proteins are well conserved and widely distributed in *H. influenzae* (Hanson *et al.*, 1992b; Frangipane *et al.*, 1994; Dunten and Mowbray, 1995; Jin *et al.*, 1996; Maciver *et al.*, 1996;), *H. ducreyi* (Elkins, 1995; Thomas *et al.*, 1998), pathogenic and commensal neisseriae (Lee, 1994; Stojiljkovic *et al.*, 1996; Richardson and Stojiljkovic, 1999), *P. multocida* (Bosch *et al.*, 2002), *A. pleuropneumoniae* (Archambault *et al.*, 2003; Srikumar *et al.*, 2004) and *A. actinomycetemcomitans* (Hayashida *et al.*, 2002). Although some Hm- and Hb-binding proteins, such as HbpA and HgbB of *P. multocida*, are immunogenic, single protein vaccines appear to have no protective properties (Cox *et al.*, 2003; Garrido *et al.*, 2003; Bosch *et al.*, 2004). Vaccines produced from protein inlcuding several Hm- and Hb-binding proteins from *P. multocida* do, however, appear to be protective (Bosch *et al.*, 2004).

1.3.4 Regulation of genes involved in iron acquisition

In response to low levels of iron in their hosts, invading bacteria produce an array of proteins involved in iron acquisition and metabolism. However, the regulator involved in the control of expression of iron-repressible proteins was unknown until Hantke (1981) described a mutant of *E. coli* that constitutively expressed proteins involved in iron uptake. The mutated gene encoding the putative regulatory protein was designated *fur* and the wild-type allele was cloned (Hantke, 1984) and sequenced (Schaffer *et al.*, 1985). The Fur protein is relatively small (~17 kDa) and has been identified in several bacterial species (see e.g., Ratledge and Dover, 2000). Fur is a homodimer with dimerization involving interactions between the C-terminal domains (Stojiljkovic and Hantke, 1995). When iron is in excess, Fe^{2+} and Fur associate, and this complex acts as a repressor by binding to a specific DNA sequence within the promoter of iron-repressible genes. The binding of the Fur-Fe^{2+} complex inhibits the binding of RNA polymerase and thereby represses the transcription of the gene (Escolar *et al.*, 1999). The DNA region bound by Fur is referred to as a Fur box and was originally determined to be a 19-bp consensus (5'ATAATGATWATCATTATC 3') composed of two 9-bp repeats separated by 1 bp (the W). This exact Fur box sequence, however, is not found in the *E. coli* genome and matches as low as 11/19 bp were identified as Fur-box sequences. This led Escolar *et al.* (1999) to suggest an alternative description for a Fur-box sequence, namely, a sequence composed of 3 repeats of a 6-bp motif (see Fig. 1.2). This model offered an explanation for the ability of Fur to bind to adjacent sites lacking Fur boxes but it did not explain how individual dimers are arranged on the DNA helix (Andrews *et al.*, 2003). To explain the corkscrew binding of the Fur dimer, Lavrrar *et al.* (2002) suggested a Fur-box model

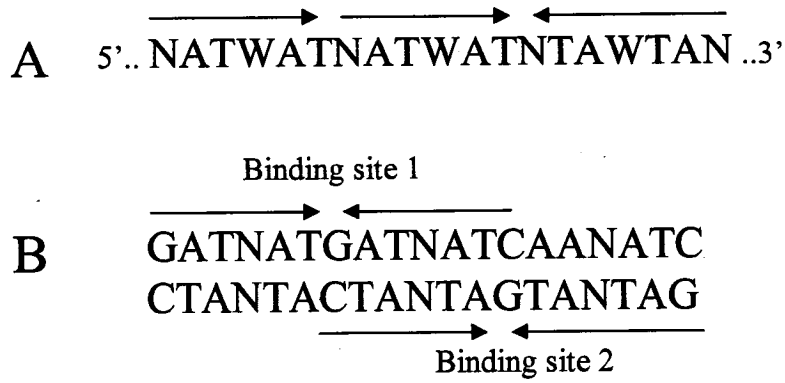


Fig. 1.2. (A) Fur-box model proposed by Escolar *et al.* (1999). The 3 NATWAT motifs are organised in 2 forward-1bp-1 reverse fashion. (B) Fur-box model proposed by Lavrrar *et al.* (2002). Binding sites for each Fur subunit of the dimer are present in the sequence and are designated binding sites 1 and 2.

containing an overlapping 13-bp (6-1-6) motif (see Fig. 1.2).

In vitro, Fur can associate with metals chemically related to iron such as Co^{2+} and Mn^{2+}; however, the *in vivo* concentration of these metals is insufficient to have a significant effect on the regulation of iron-repressible genes (Andrews *et al.*, 2003). In the presence of metals, the affinity of Fur for DNA increases ~1000 fold (Andrews *et al.*, 2003). The binding of Fe^{2+} occurs at the C-terminal domain (Hantke, 2001).

Virtually all the iron-uptake systems of Gram-negative bacteria that have been examined are regulated directly by Fur (Braun *et al.*, 1998). Fur is the main element involved in the maintenance of iron homeostasis; however, other iron regulator proteins have been identified. In *Bradyrhizobium japonicum*, Irr is similar to Fur and Irr regulates the Hm biosynthesis pathway (Hamza *et al.*, 1998). Unlike Fur, Irr is active under iron-restricted conditions. *B. japonicum* also possesses a Fur homologue and the Fur box is recognized by Irr (Friedman and O'Brian, 2003). In Gram-positive organisms, Fur homologues have been identified in species such as *Bacillus subtilis* (Hantke, 2001). High G-C Gram-positive bacteria also possess a protein that is structurally similar to Fur. This protein was first identified in *Corynebacterium diphtheriae* and was named the diphtheria toxin regulator (DtxR). DtxR was found to promote the production of diphtheria toxin under iron restricted conditions (Boyd *et al.*, 1990). DtxR and DtxR-like proteins also regulate the expression of genes homologous to those regulated by Fur (Hantke, 2001).

1.3.5 The TonB system

The outer membrane of Gram-negative bacteria is a semi-permeable barrier that allows molecules smaller than 600 Da to cross (Braun and Killman, 1999; Koebnick *et al.*, 2000). For large molecules, which include most iron complexes, high-affinity

receptors and active transport are required. The challenge faced by a Gram-negative bacterium is that the energy required in the periplasm and outer membrane must be supplied from the cytoplasm (Braun and Killman, 1999). Significantly, in 1943, Luria and Delbruck reported the isolation of an *E. coli* mutant, named TonB (for T-one), that was resistant to phage T1 (Postle, 1990). Phage T1 was capable of binding to the *tonB* mutant strain of *E. coli*, but the phage nucleic acid was not actively imported into the cell (Postle, 1990; Braun, 1995; Braun *et al.*, 1998). Later, it was demonstrated that the TonB mutant could not transport siderophores and vitamin B_{12} (Postle, 1990; Braun *et al.*, 1998). The protein capable of transducing energy from the cytoplasm to the outer-membrane receptor is encoded by *tonB* and has a molecular mass of ~23 kDa (Braun, 1995; Moeck and Coulton, 1998). TonB, or a functional homologue, is an essential component of iron acquisition systems of several Gram-negative pathogens as established in TonB knock-out mutant studies using *Bordetella* spp. (Nicholson and Beall, 1999; Pradel *et al.*, 2000), *E. coli* (Braun, 1995; Torres *et al.*, 2001), *H. influenzae* (Jarosik *et al.*, 1994, 1995), *Neisseria* spp. (Biswas *et al.*, 1997; Stojiljkovic and Srinivasan, 1997) and *P. aeruginosa* (Takase *et al.*, 2000).

Although TonB can function independently, the energy transfer activity is at its peak efficiency when TonB is complexed with two accessory proteins, ExbB (~26 kDa) and ExbD (~15 kDa) (Braun, 1995; Moeck and Coulton, 1998). Two TonB proteins may form a cytoplasmic membrane complex with 14 or 15 ExbB and 4 to 5 ExbD to yield a complex that can be as large as 520 kDa (Held and Postle, 2002). The exact mechanism of energy transduction has yet to be elucidated; however, several of the interactions between TonB and outer membrane receptors have been described. TonB is anchored in

the cytoplasmic membrane via its N-terminus and two proline-rich regions create a rod-like structure that spans the periplasmic space. The C-terminus of TonB interacts and associates preferentially with receptors residing within the outer membrane and possessing bound ligand (Moeck and Coulton, 1998; Howard *et al.*, 2001; Moeck and Lettellier, 2001). TonB-dependent outer-membrane receptors possess a conserved N-terminal motif, called the TonB box, that is generally thought to interact physically with TonB (Braun, 1995; Moeck and Coulton, 1998).

TonB homologues have been identified in several bacterial species and TonB systems with a dedicated role have also been identified. For example, *A. pleuropneumoniae* and, probably, the closely-related species, *A. suis,* possesses two TonB systems, one (TonB1) associated specifically with acquisition of iron from Tf and the other (TonB2) involved more generally in TonB-dependent functions (Beddeck *et al.*, 2004). Other bacterial species with a role-specific TonB include *Campylobacter jejuni* (Guerry *et al.*, 1997; Parkhill *et al.*, 2000), *Vibrio cholerae* (Seliger *et al.*, 2001; Mey and Payne, 2003), *S. marcescens* (Paquelin *et al.*, 2001) and *P. aeruginosa* (Zhao and Poole, 2000). Redundant TonB proteins and TonB systems have also been recognized in some species (Tomb *et al.*, 1997; Alm *et al.*, 1999; Desai *et al.*, 2000). While the reason(s) for the duplication of TonB proteins/systems is not clearly understood, a role in pathogenicity has been suggested (Beddeck *et al.*, 2004).

1.4 Aims of this research project

Several members of the *Pasteurellaceae* and *Neisseriaceae* are known to acquire Hb- and Tf -bound iron in a contact-dependent manner. While *H. somni* has been shown to acquire iron from Tf by means of siderophore-independent receptor-mediated

mechanisms similar to those found in other members of the *Pasteurellaceae* (Yu *et al.*, 1992; Yu and Schryvers, 1994; Ekins and Niven, 2001; Ekins and Niven, 2002; Ekins *et al.*, 2004a), no information is available regarding the acquisition of Hb-bound iron. The purpose of this research project was to determine if *H. somni* is capable of acquiring Hb-bound iron and, if so, to characterize some of the components involved in this process.

Chapter 2 - Materials and Methods

2.1 Organisms and growth conditions

H. somni strains 9L, 3384Y, 642A, 714 and 5688T were provided by Dr. R.L. Walker (University of California at Davis) and were described by Walker *et al.* (1985); strains 649, 2336 and 8025 were from Dr. L.B. Corbeil (University of California at San Diego). *A. suis* strain SO4 was provided by Dr. S. Rosendal (University of Guelph). For *H. somni,* the growth medium was HEPES-buffered tryptone-yeast extract (TYE-H; 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 50 mM HEPES, 63 mM NaCl, 10 mM KCl, 10 mM Na_2HPO_4, 10 mM glucose, NaOH to pH 7.8) supplemented immediately before inoculation with thiamine monophosphate (TMP; to 10 μg/ml), L-cysteine (to 0.5 mg/mL) and $NaHCO_3$ (to 10 mM); this medium is referred to as supplemented TYE-H (sTYE-H) (Ekins and Niven, 2001). For *A. suis*, the growth medium was TYE-H supplemented with $NaHCO_3$ (to 10 mM). Incubation was at 37°C with shaking on a gyratory shaker (150 rpm for *H. somni*; 200 rpm for *A. suis*); growth was monitored turbidimetrically at 660 nm (Gilford Stasar II spectrophotometer, Gilford Instrument Laboratories Inc.). Cultures were stored frozen at –80°C as small aliquots (~ 1.5 ml) supplemented with 15% (w/v) glycerol.

For growth assays, *H. somni* was cultured (1% (v/v) inoculum) in 25-ml vols (125-ml screw-capped Nalgene flasks) of sTYE-H containing 50 μM ethylenediamine di-*o*-hydroxyphenylacetic acid (EDDA; Sigma; added prior to autoclaving) and where appropriate, supplemented, immediately before inoculation, with either Hb (to 80 μg/ml)

or Hm (to 5 μM). *A. suis* (0.1% (v/v) inoculum) was cultured similarly except that TYE-H was used instead of sTYE-H.

For the preparation of membranes, *H. somni* was grown in 200-ml vols of either sTYE-H (iron-replete conditions), sTYE-H with bovine Hb (to 80 μg/ml), sTYE-H with EDDA (12 – 14 μM; iron-restricted conditions) or sTYE-H containing EDDA (50 μM) and bovine Hb (to 80 μg/ml).

For investigation of phase variation, *H. somni* strains 649, 2336 and 8025 were grown to late exponential phase in 25-ml vols of sTYE-H containing EDDA and Hb, as above. Samples (1 ml) were removed from these cultures and serially-diluted using sterile saline as the diluent. The resulting dilutions (0.1-ml vols) were spread on solid sTYE-H (1.6% agar). Plates were incubated for 48 h at 37°C in an atmosphere enriched with CO_2 (BBL GasPak). Isolated colonies were resuspended in 25 μL of 10 mM HEPES (pH 7.4 with KOH). These suspensions (5-μL vols) were then used as DNA templates in PCR.

2.2 Hm, Hb and conjugates

Hm and bovine, ovine, porcine and human Hbs were from Sigma. Hm was dissolved in 20 mM NaOH, 50% ethanol to give a final concentration of 4 mM. For growth assays, Hb (100 mg) was dissolved in 40 mM Tris, 2 mM $NaHCO_3$, HCl to pH 7.4 (10 ml), dialysed (Spectra/Por, Spectrum Medical Industries; 6000-8000 MWCO) against 5 mM Tris, 145 mM NaCl, HCl to pH 7.4 (1-l vols with 3 changes) and filter sterilized (0.22 μm pore size); biological activity was tested by means of growth assays using *A. suis* strain SO4 (*A. suis* can use all four Hbs as iron sources for growth; F. Bahrami, personal communication). For biotinylation, 10 mg of Hb was dissolved in 10

ml of phosphate buffered saline (PBS; 0.17 M NaCl, 3.4 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4). The mixture was dialysed against PBS, as above. Hbs were biotinylated using the ECL protein biotinylation module (Amersham Pharmacia Biotech). Briefly, 2.5 mg of Hb was mixed with the biotinylation reagent and incubated with gentle shaking (room temperature, 1 h). The biotinylated Hb was purified using a Sephadex G-25 column with the total eluate being collected.

2.3 Preparation of membrane fractions

Membrane fractions were prepared from late exponential phase organisms essentially as described by Niven *et al.* (1989). Briefly, the organisms from each 200-ml volume of culture were collected by centrifugation (10 000 × g, 10 min, 4°C), washed twice with culture volumes of 145 mM NaCl (centrifuge as above), resuspended in 10 mL of 10 mM HEPES and frozen at –20 °C. Such suspensions were thawed, the organisms disrupted by sonication, and unbroken cells and large debris sedimented by centrifugation (16 000 × g, 10 min, 4°C). The resulting supernatant was subjected to high-speed centrifugation (180 000 × g, 60 min, 4°C) and the pellet resuspended in 10 ml of 10 mM HEPES. A 5-ml volume of this suspension (total membranes) was removed, dispensed as ~0.5 ml aliquots and stored frozen (–20 °C); to the remainder was added 5 ml H_2O and 10 ml of a sarkosyl solution (2% (w/v) in 10 mM HEPES) and following a 30-min incubation (room temperature), the resulting sarkosyl-treated membranes were harvested by centrifugation (180 000 × g, 60 min, 4°C). These membranes were resuspended with H_2O (~ 20 ml), harvested again (180 000 × *g*, 60 min, 4°C) and resuspended in a small volume of H_2O (0.5 ml); the resulting suspension (outer membranes) was stored at –20°C.

2.4 SDS-PAGE

Total membranes and outer membranes were analysed by means of SDS-PAGE (5.2% stacking gel and 12% running gel) as described by Ekins and Niven (2001). The electrode buffer was 0.1% SDS, 25 mM Tris, 192 mM glycine and electrophoresis (16 h, 10 mA per gel) was carried out using a Protean II electrophoresis system (Bio-Rad). Separated polypeptides were visualized by means of silver staining (Harlow and Lane, 1988).

2.5 Hb-binding assays

Hb-binding assays, using intact cells of *H. somni*, biotinylated Hb as ligand and streptavidin-HRP (Pierce) as detection agent, were carried out essentially as described by Ekins and Niven (2001). Intact cells were from 25-ml late exponential phase cultures grown under the conditions that were used to produce cells for the preparation of membranes (see Section 2.1). The organisms were harvested by centrifugation (14 000 × *g*, 1 min, room temperature), washed twice with 145 mM NaCl (14 000 × *g*, 1 min, room temperature) and resuspended in Tris-buffered saline (TBS; 145 mM NaCl, 100 mM Tris-HCl, pH 7.4) such that the OD_{660} was 1.0. Intact cells (50 µl – 100 µl) were applied to a nitrocellulose membrane held in a dot blot apparatus (Minifold I; Schleicher and Schuell). The membrane was air dried (37°C, 30 min) and then incubated (37°C, 1 h), with gentle agitation, in TBS containing Tween 20 (0.15% v/v; TTBS). Biotinylated Hbs (250 ng per well) were added to the appropriate well and samples were incubated at 37°C for 1 h. The Hb solution was removed, the sheet washed thrice with TBS (10 min, 37°C, gentle

agitation), incubated with streptavidin-HRP (50 ng), washed thrice with TBS and then developed with the 4-chloro-1-naphthol/H_2O_2 reagent described by Niven *et al.* (1989). For competition assays, biotinylated bovine-Hb (250 ng per well) was mixed with the appropriate native Hb (0.1 mg per well), or with TBS, and the binding assays then carried out as described above.

In some experiments, intact cells were first treated with trypsin (Sigma), as described by Wong and co-workers (1994), with some modifications. Briefly, suspensions of intact cells (1-ml vols) were subjected to centrifugation (14 000 × g, 1 min, room temperature) and the harvested cells washed twice with 145 mM NaCl (14 000 × g, 1 min, room temperature) and resuspended using 1-ml volumes of Hank's balanced buffer solution (0.4 g/l KCl, 0.06 g/l KH_2PO_4, 0.35 g/l $NaHC0_3$, 8 g/l NaCl, 0.05 g/l Na_2HPO_4, 1 g/l glucose, NaOH to pH 7.5). Trypsin was added (0 to 100 μg) and the resulting suspensions were incubated for 45 min at 37°C with gentle shaking. Reactions were stopped by adding an equimolar concentration of soybean trypsin inhibitor (Sigma) and the resulting mixtures were incubated for 5 min at 37°C. Finally, the cells were centrifuged (14 000 × g, 1 min, room temperature), washed twice with 145 mM NaCl (14 000 × g, 1 min, room temperature) and resuspended in TBS (1-ml vols).

2.6 DNA isolation, amplification and sequencing

Genomic DNA was isolated from *H. somni* strains 649, 9L and 3384Y using a DNeasy Tissue Kit (Qiagen) or Genomic-tip 500/G columns and the recommended buffer system (Qiagen). Primers for PCR (HsHgF1 and HsHgR1; see Table 2.1) were designed based on the incomplete *H. somni* strain 2336 genome sequence

(http://microgen.ouhsc.edu/h_somnus/h_somnus_home.htm) and on highly conserved regions of Hb-binding proteins of the neisseriae and various *Pasteurellaceae*. Custom-made primers were used in PCR with *H. somni* genomic DNA. The reaction mixture (50 μL) contained 2 μL of genomic DNA template, 0.5 μM upstream primer, 0.5 μM downstream primer, 0.2 mM dNTP, 3 mM $MgCl_2$, 5 μL of 10 × PCR Buffer and 2.5 U of *Taq* polymerase. The PCR mixture was subjected to 3 min at 95°C, 30 cycles of 1 min at 95°C, 1 min at 54°C and 2 min at 72°C, and a final 2 min at 72°C using a thermocycler. The resulting PCR products were purified using a QIAquick PCR Purification kit (Qiagen) and sequenced using the custom-made primers and a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems); samples for sequencing were purified using AutoSeq G-50 columns (Amersham Pharmacia Biotech). Sequencing reactions were analyzed by the Applied Biotechnology Laboratory (McGill University, Macdonald Campus) using an ABI 310 Genetic Analyzer (Applied Biosystems). In the studies relating to phase variation, primers HmHoRD2 and HBHSF1 (see Table 2.1) and cell suspension as DNA template were used in PCR. The PCR products were sequenced and analyzed by Genome Quebec Innovation Centre (McGill University) using an ABI PRISM 3730XL DNA Analyzer (Applied Biosystems). Sequence data were assembled using Sequencher 3.0 (Gene Codes). ORF searches were performed using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/) and sequence analyses were performed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Motif and pattern searches were done using ScanProsite (http://ca.expasy.org/tools/scanprosite) and InterProScan (http://www.ebi.ac.uk/InterProScan/).

Table 2.1. *hugX*-, *hugZ*- and *hgbA*-specific primers

Primer	Description (coordinates)[a]	Sequence (5' → 3')
HsHgF1	2336 *hgbA* Forward (1739-1762)	AGATGGTTTACACCAAGCGGAAAC
HsHgR1	2336 *hgbA* Reverse (2073-2096)	GTTCTGTAGTTCATGACCATCACG
HmHoRD2	*hgbA* Reverse (1757-1780)	AAGCCCTGAGAACTCAACGTTTCC
HBHSF1	*hugZ* Forward (1299-1322)	GGTTTTGGTCAAGCTTTCCAAGTG
HugXF2	*hugX* Forward (775-798)	CTTGGTCGTGATGAACAACGTCAG
HbHoRD1	*hgbA* Reverse (1488-1511)	GGAAGCGGAGTTAGTTTGAGCATC

[a] Base pair coordinates refer to the numbering given for *hugX*, *hugZ* and *hgbA* of strain 649 (GenBank Accession no. AY380576).

2.7 RNA isolation and RT-PCR

H. somni strain 649, grown under iron-replete and iron-restricted conditions (see Section 2.1), was harvested as described by Bahrami *et al.* (2003). Briefly, 1-ml volumes of each culture were centrifuged (14 000 × g, 1 min, room temperature), washed with 1 ml PBS (centrifugation as above) and resuspended with 0.1 ml PBS followed by 0.9 ml RNA*later* RNA Stabilization Reagent (Qiagen). RNA was isolated using the RNeasy Mini Kit (Qiagen) and further purified as described by Bahrami *et al.* (2003). RT-PCR was carried out with the OneStep RT-PCR Kit (Qiagen), 400 ng of the appropriate RNA sample, or an aliquot of DNA (positive control), and primers HugXF2 and HbHoRD1. PCR, with the appropriate primers and RNA (400 ng) as template, served as negative controls. Products were analysed by means of gel electrophoresis (1% agarose, TAE buffer) and staining with ethidium bromide.

2.8 Estimation of protein concentrations

Protein concentrations were estimated using the method of Gornall *et al.* (1949) and bovine serum albumin (fraction V powder; Boehringer-Mannheim) as the standard or using a *DC* Protein Assay Kit (Bio-Rad).

2.9 Imaging

Images of dot blots and gels were obtained using an AlphaImager 1200 documentation and analysis system and AlphaEase software (Alpha Innotech).

2.10 Nucleotide sequence accession numbers

The sequences of the genes described in this study were submitted to GenBank and assigned Accession numbers AY380576 (strain 649), AY383479 (strain 3384Y) and AY501999 (strain 9L).

Chapter 3 - Results

3.1 *H. somni* can acquire iron from bovine Hb but not from ovine, porcine or human Hbs

Five strains of *H. somni* were investigated for their ability to acquire iron from Hb. All strains (649, 2336 and 8025) previously referred to as *H. somnus*, were capable of utilizing iron from bovine Hb but not from ovine, porcine or human Hb or from Hm. However, the times required for strains 2336 and 8025 to complete their growth cycles were noticeably longer than that required by strain 649 (Figs. 3.1, 3.2 and 3.3). Two strains (9L and 3384Y) previously referred to as *H. ovis*, failed to use any of the Hbs or Hm as an iron source for growth (Figs. 3.4 and 3.5). Three other ovine strains (642A, 714 and 5688T) were also tested for their ability to acquire iron from bovine and ovine Hbs and from Hm; none of these organisms could acquire iron from these iron sources. Stojiljkovic *et al* (1996) reported that *N. meningitidis* acquires iron preferentially from human Hb. However, this preference was not noticeable when the concentrations of the non-human Hbs were approximately twice that of the human. Increasing the concentration of the Hbs to 160 μg/ml and Hm to 10 μM and 25 μM did not have any noticeable impact on the growth of *H. somni* strain 649.

3.2 *H. somni* can bind bovine, ovine and human Hbs by means of the same iron-repressible receptor(s)

Solid phase binding assays were used to investigate the binding of Hbs by intact cells of *H. somni*. When grown under iron-restricted, but not iron- replete, conditions in the presence or absence of Hb, strain 649 was found to be capable of binding bovine,

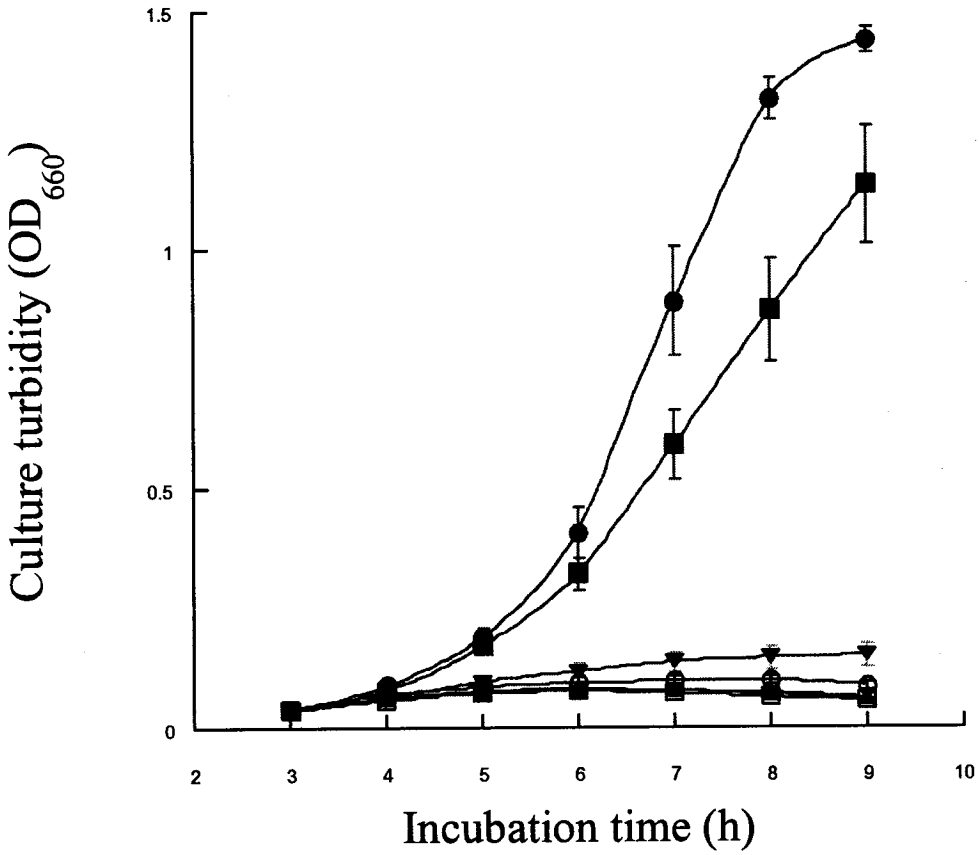


Fig. 3.1. Growth kinetics of *H. somni* strain 649 grown under iron-replete (●) and iron-restricted (□) conditions, and under iron-restricted conditions in the presence of either bovine (■), ovine (○), human (▼) or porcine (△) Hb or Hm (▲). Culture turbidity values are the means of triplicate values from two independent experiments.

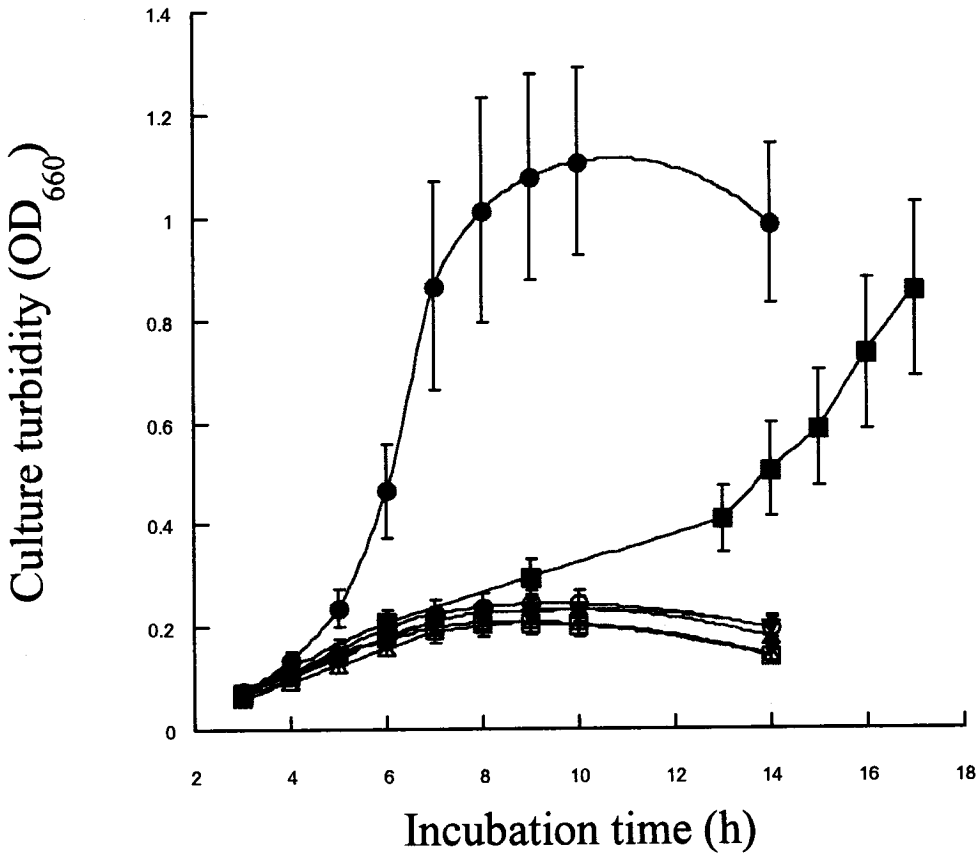


Fig. 3.2. Growth kinetics of *H. somni* strain 2336 grown under iron-replete (●) and iron-restricted (□) conditions, and under iron-restricted conditions in the presence of either bovine (■), ovine (○), human (▼) or porcine (△) Hb or Hm (▲). Culture turbidity values are the means of triplicate values from two independent experiments.

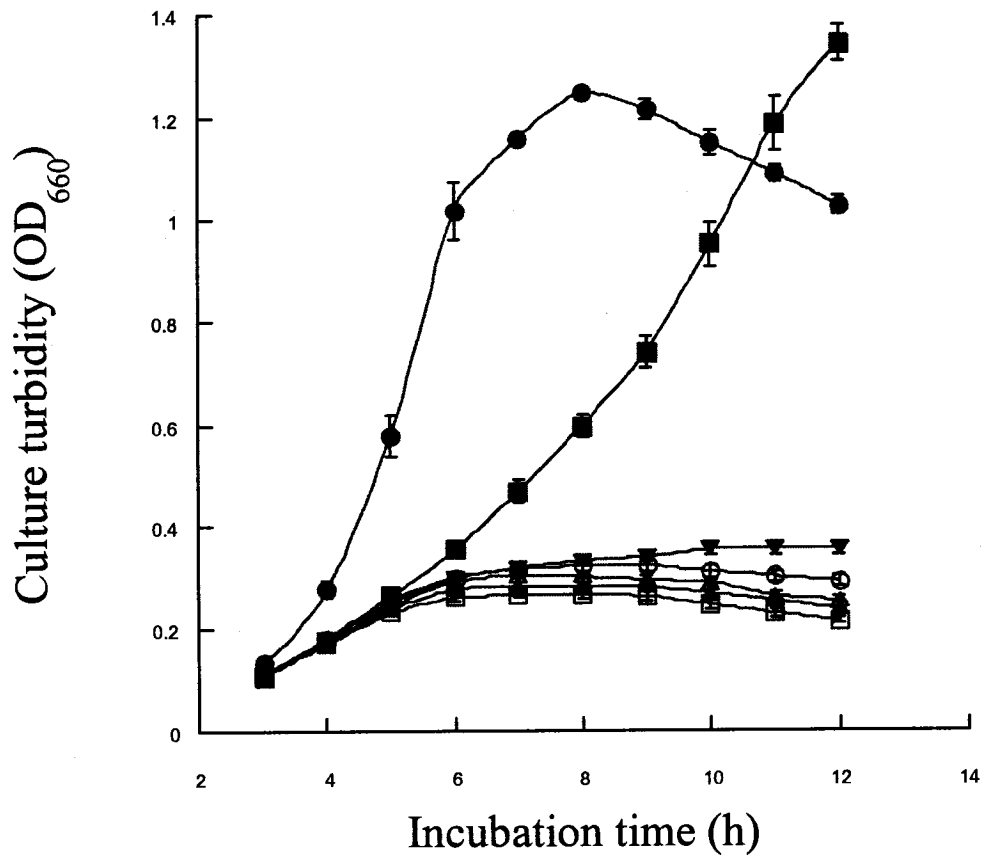


Fig. 3.3. Growth kinetics of *H. somni* strain 8025 grown under iron-replete (●) and iron-restricted (□) conditions, and under iron-restricted conditions in the presence of either bovine (■), ovine (○), human (▼) or porcine (△) Hb or Hm (▲). Culture turbidity values are the means of triplicate values from two independent experiments.

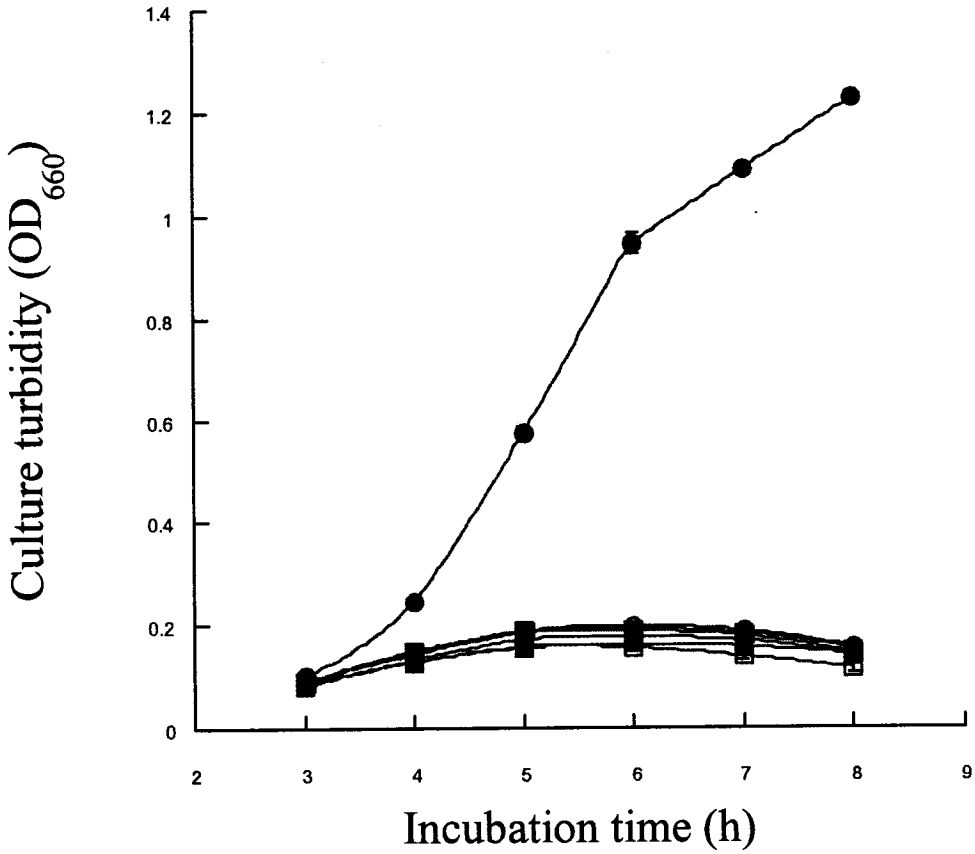


Fig. 3.4. Growth kinetics of *H. somni* strain 9L grown under iron-replete (●) and iron-restricted (□) conditions, and under iron-restricted conditions in the presence of either bovine (■), ovine (○), human (▼) or porcine (△) Hb or Hm (▲). Culture turbidity values are the means of triplicates.

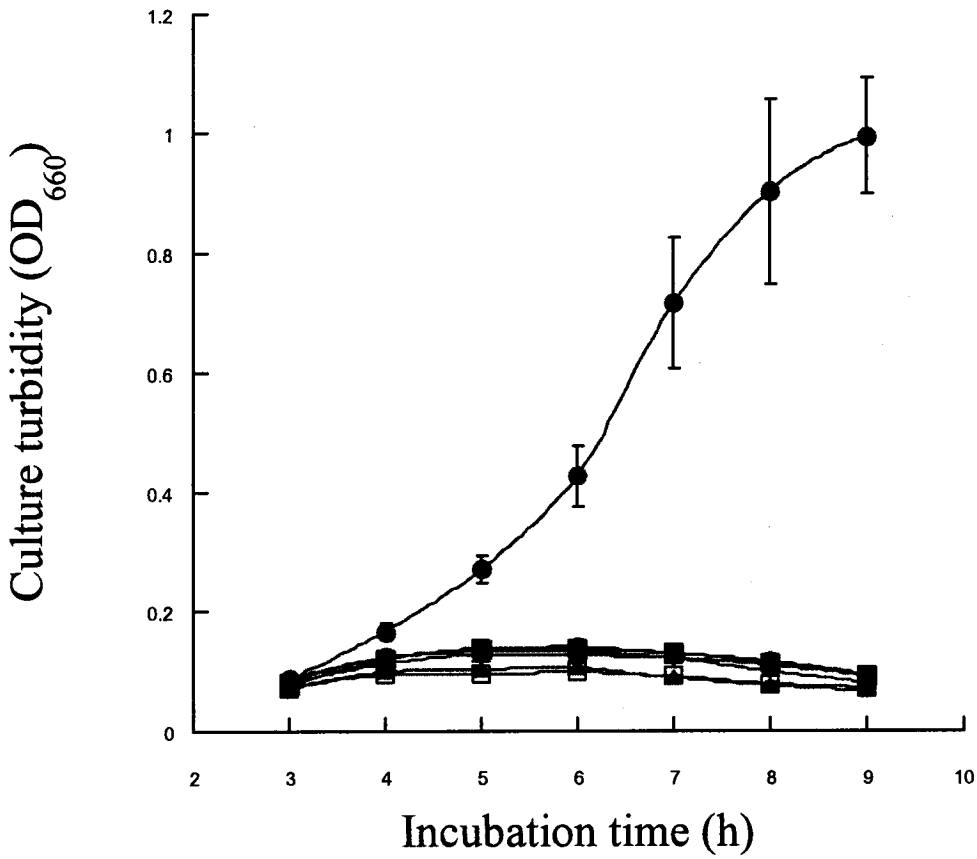


Fig 3.5: Growth kinetics of *H. somni* strain 3384Y grown under iron-replete (●) and iron-restricted (□) conditions, and under iron-restricted conditions in the presence of either bovine (■), ovine (○), human (▼) or porcine (△) Hb or Hm (▲). Culture turbidity values are the means of triplicates.

ovine and human Hbs (Fig. 3.6). Analogous results were obtained with strains 2336 and 8025 but only if the organisms were grown under iron-restricted conditions in the presence of Hb (Fig. 3.7). Competition assays revealed that bovine, ovine and human Hbs, but not porcine Hb, are able to compete effectively with biotinylated bovine Hb for binding sites on all three strains (Fig. 3.8). However, strain 649 was also shown to bind this Hb, albeit with reduced efficiency, even after treatment with trypsin (Fig. 3.9) suggesting that biotinylated ovine Hb may be bound by some non-protein component.

3.3 *H. somni* can produce an iron-repressible, ~120-kDa outer-membrane protein

Total membranes and outer membranes derived from strains 649, 2336 and 8025 were subjected to SDS-PAGE (Figs. 3.10, 3.11 and 3.12). A polypeptide with an estimated molecular mass of ~120 kDa was detected in total and outer membranes from all three strains but only if the membranes were from organisms grown under the conditions that promoted the expression of Hb binding activity. Unfortunately, the ~120-kDa polypeptides could not be affinity-isolated using any of the methods described by Ricard *et al.* (1991), Elkins (1995) or Jin *et al.* (1996), with or without modifications. Also, transfer of the separated (SDS-PAGE) polypeptides from gels to nitrocellulose membranes, followed by reaction with biotinylated bovine Hb and streptavidin-HRP, yielded no significant results.

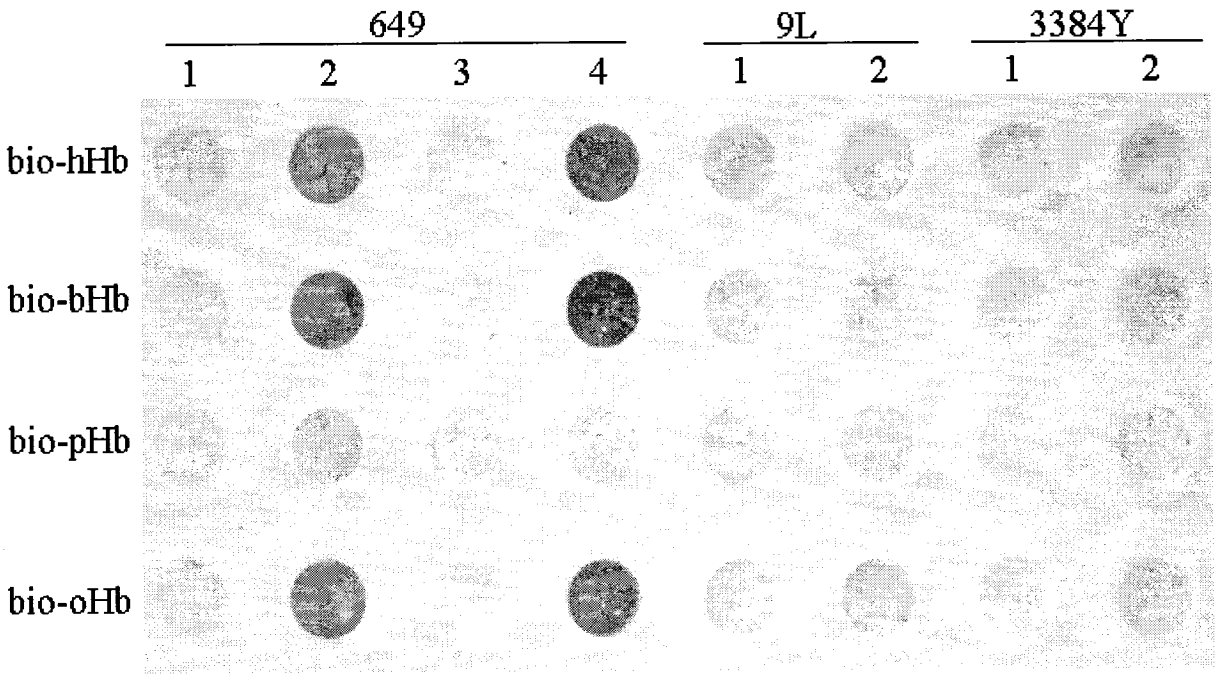


Fig. 3.6. Dot blot demonstrating binding of the indicated Hbs by intact cells of *H. somni* strains 649, 9L and 3384Y grown under iron-replete (1) and iron-restricted (2) conditions, and under iron-replete (3) and iron-restricted (4) conditions in the presence of bovine Hb. The abbreviations are as follows: o = ovine, b = bovine, p = porcine, h = human, bio = biotinylated. This figure is representative of several assays.

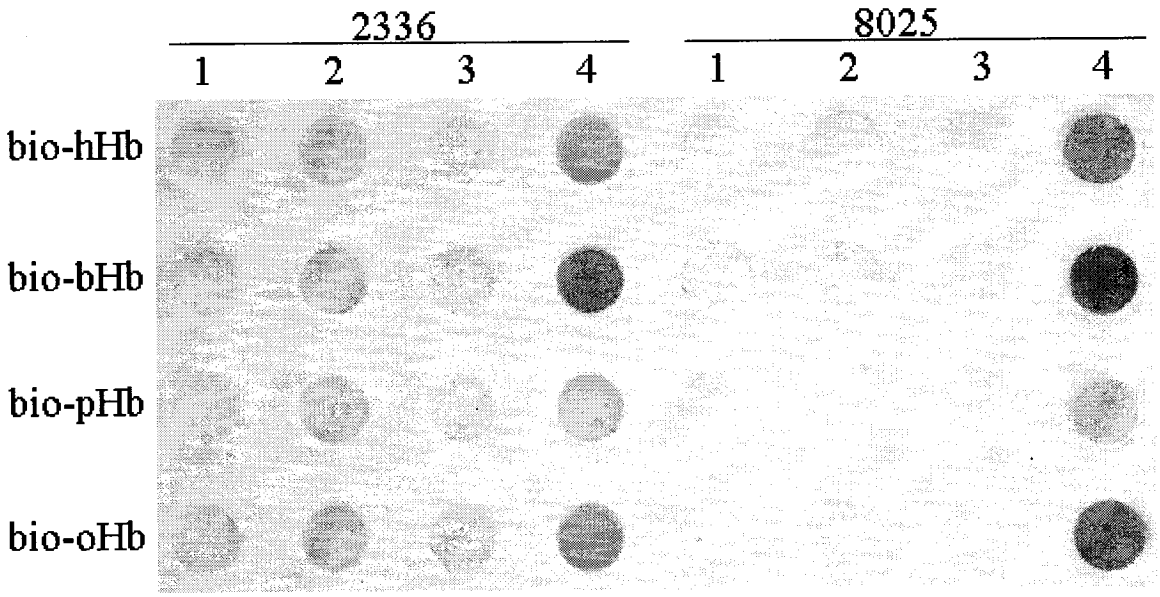


Fig. 3.7. Dot blot demonstrating binding of the indicated Hbs by intact cells of *H. somni* strains 2336 and 8025 grown under iron-replete (1) and iron-restricted (2) conditions, and under iron-replete (3) and iron-restricted (4) conditions in the presence of bovine Hb. The abbreviations are as follows: o = ovine, b = bovine, p = porcine, h = human, bio = biotinylated. This figure is representative of several assays.

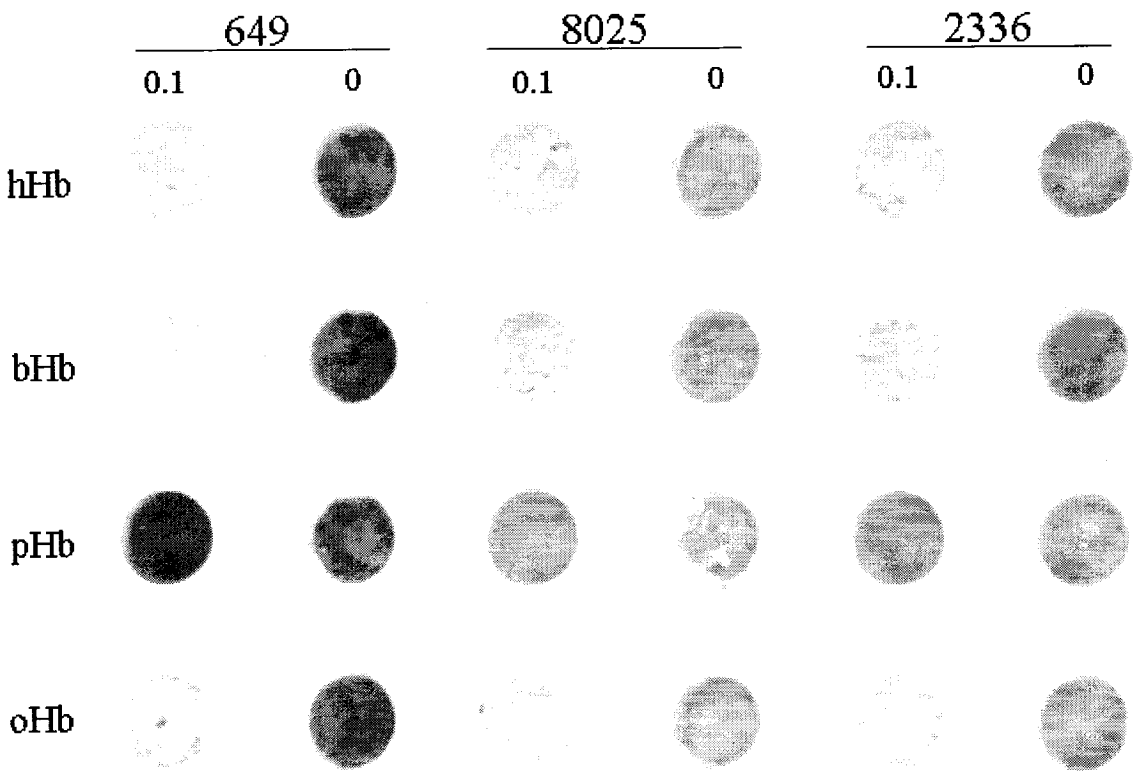


Fig. 3.8. Dot blot demonstrating competition between native Hbs and biotinylated bovine Hb for Hb binding sites on intact cells of *H. somni* grown under iron-restricted conditions in the presence of bovine Hb. The presence (0.1 (mg)) and absence (0) of competing Hbs are indicated. The Hbs are abbreviated as follows: o = ovine, b = bovine, p= porcine, h = human. This figure is representative of several assays.

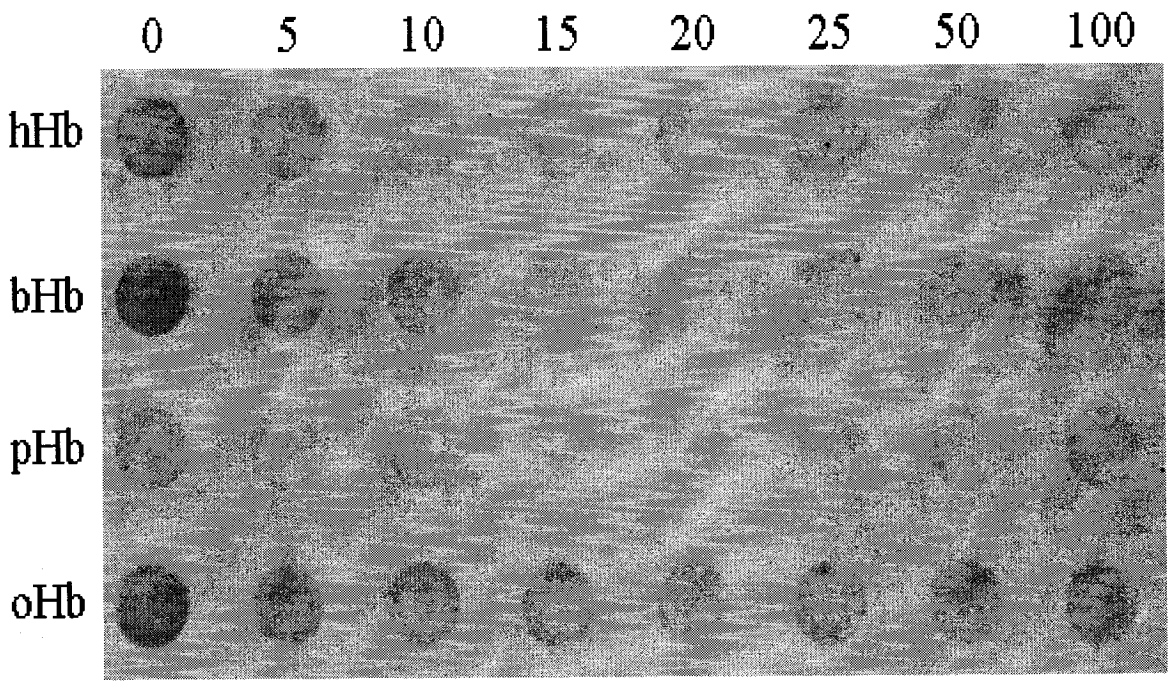


Fig. 3.9. Dot blot demonstrating binding of the indicated Hbs by trypsin-treated cells of *H. somni* strain 649 grown under iron-restricted conditions in the presence of bovine Hb. The abbreviations are as follows: o = ovine, b = bovine, p = porcine, h = human, bio = biotinylated. Numbers indicate the concentration (μg/ml) of trypsin used. This figure is representative of several assays.

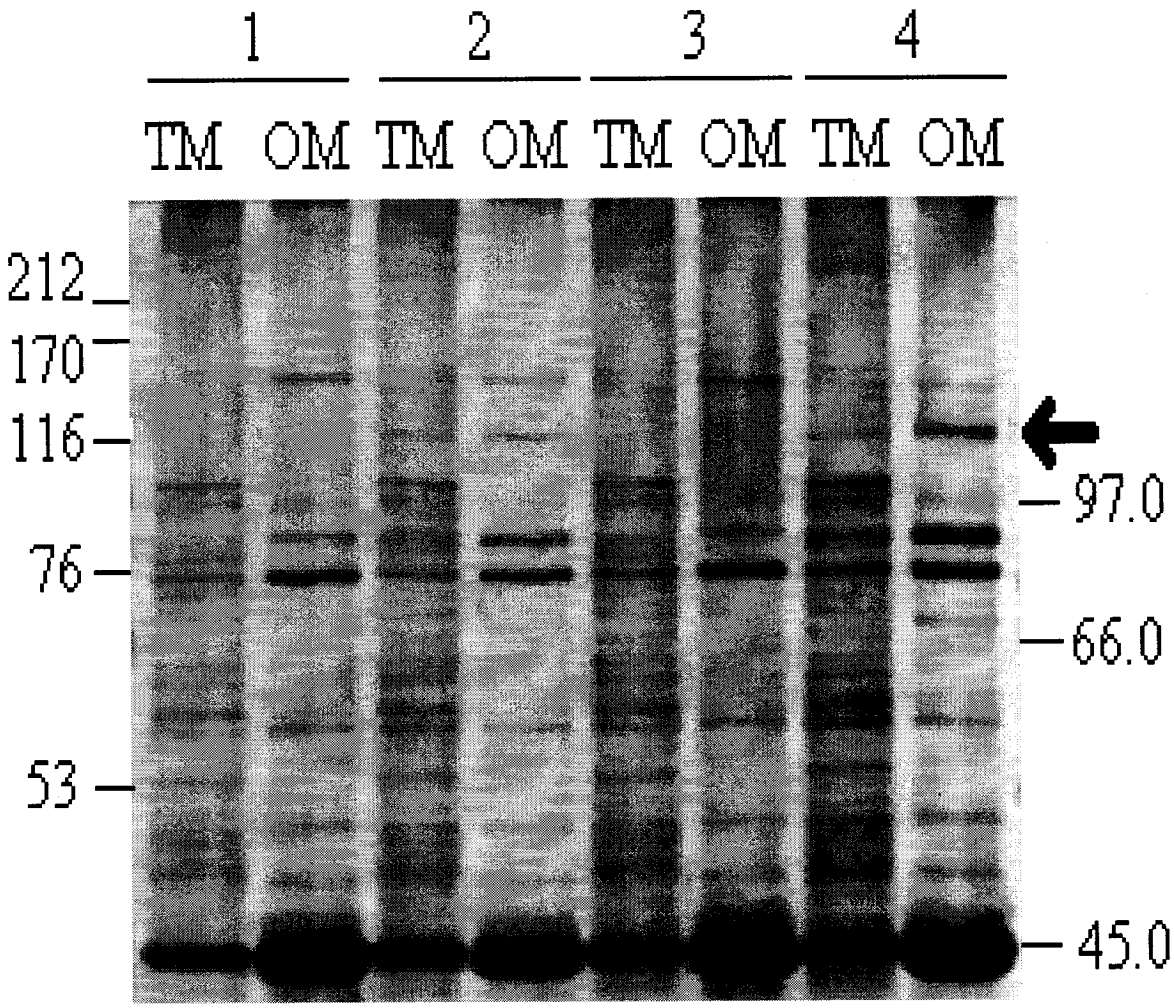


Fig. 3.10. SDS-PAGE of total membranes (TM) and outer membranes (OM) from *H. somni* strain 649 grown under iron-replete (1) and iron-restricted (2) conditions, and under iron-replete (3) and iron-restricted (4) conditions in the presence of bovine Hb. The numbers refer to the sizes (kDa) and positions of protein standards. The arrow indicates the iron-repressible, ~120-kDa outer-membrane polypeptide.

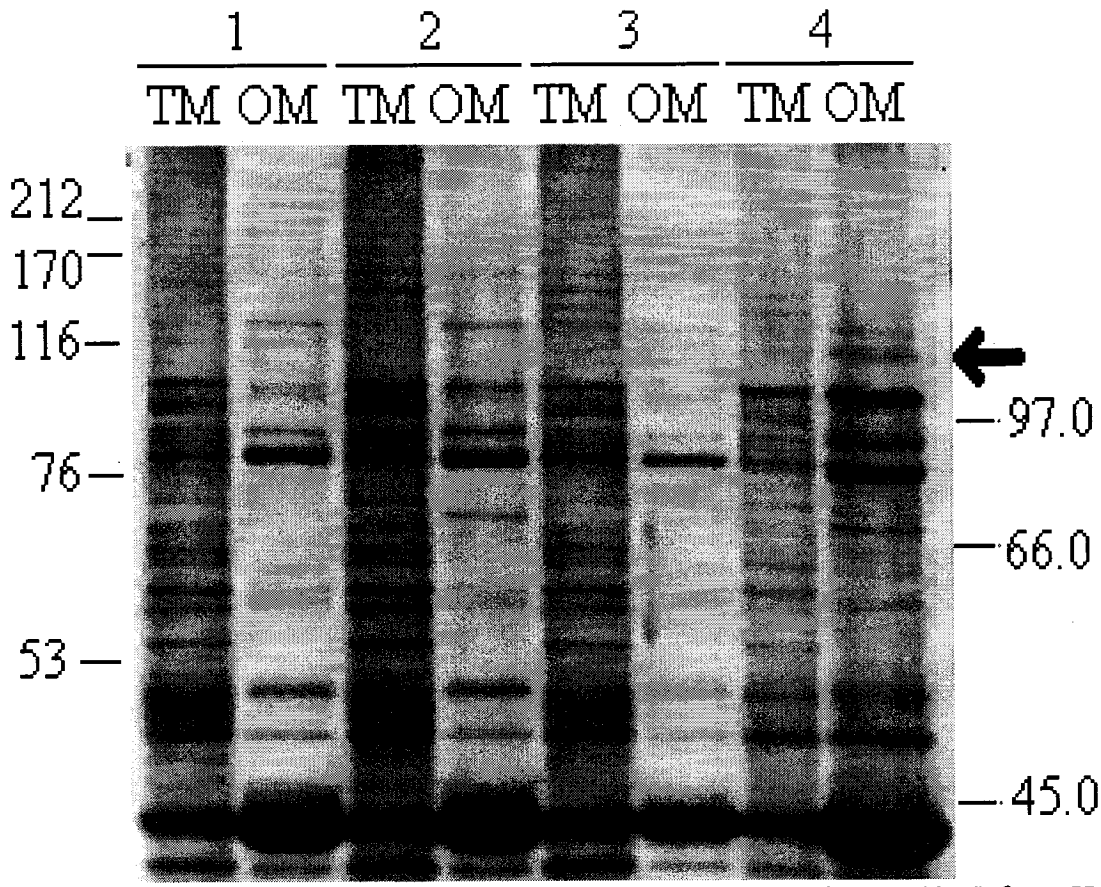


Fig. 3.11. SDS-PAGE of total membranes (TM) and outer membranes (OM) from *H. somni* strain 2336 grown under iron-replete (1) and iron-restricted (2) conditions, and under iron-replete (3) and iron-restricted (4) conditions in the presence of bovine Hb. The numbers refer to the sizes (kDa) and positions of protein standards. The arrow indicates the iron-repressible, ~120-kDa outer-membrane polypeptide.

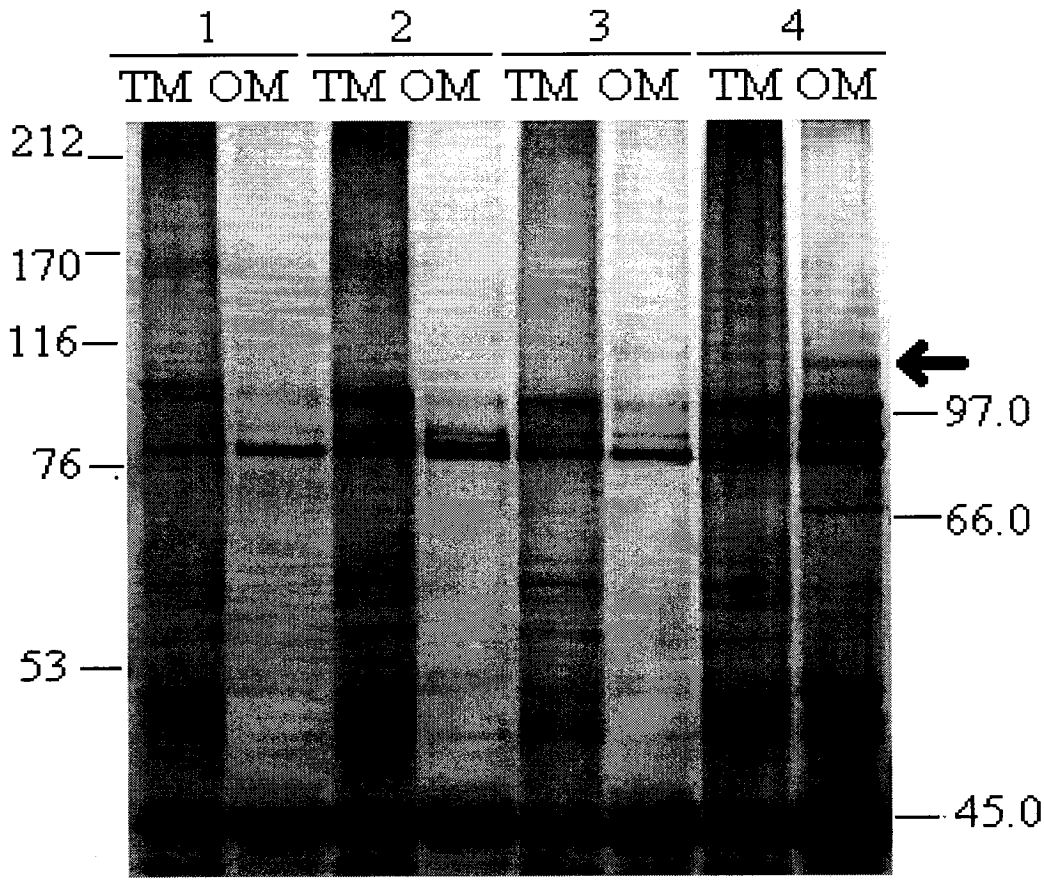


Fig. 3.12. SDS-PAGE of total membranes (TM) and outer membranes (OM) from *H. somni* strain 8025 grown under iron-replete (1) and iron-restricted (2) conditions, and under iron-replete (3) and iron-restricted (4) conditions in the presence of bovine Hb. The numbers refer to the sizes (kDa) and positions of protein standards. The arrow indicates the iron-repressible, ~120-kDa outer-membrane polypeptide.

3.4 *H. somni* possesses *hugX*, *hugZ* and *hgbA* homologues that are co-transcribed and iron-regulated.

Primers based on the incomplete *H. somni* strain 2336 genome sequence allowed the amplification of an ~300-bp fragment from all eight strains. Sequencing of the fragments that were amplified from DNA from three representative strains (649, 9L and 3384Y) revealed homology with genes that encode Hb-binding proteins. Regions upstream and downstream of the ~300-bp fragment from strains 649, 9L and 3384Y were then sequenced using cycles of primer walking and PCR. ORF analyses revealed that each sequence contained four putative ORFs, three complete and one incomplete (see Fig. 3.13). The incomplete ORFs, encoding either 47 amino acids (strain 649) or 50 amino acids (strains 9L and 3384Y), were found to be representative of *ftsJ* which encodes a 23S rRNA methyltransferase involved in heat-shock responses and cell cycle control (Tomoyasu et al, 1993; Bügl et al, 2000). Interestingly, the polypeptides encoded by these incomplete ORFs have counterparts, with high similarity (>85%), in proteins found in a variety of organisms including *P. multocida*, *H. influenzae*, *H. ducreyi*, *E. coli*, *Shigella flexneri* and *Salmonella enterica* serovars Typhi and Typhimurium. The other ORFs were identified as homologues of *P. shigelloides hugX* and *hugZ* and *P. multocida hgbA* and were found to be organized in a manner similar to that exhibited by PM299, PM300 and *hgbA* of *P. multocida* (see Bosch *et al.*, 2002). HugX and HugZ have been shown to play a role in utilization of Hm-bound iron (Henderson *et al.*, 2001). HugZ is similar to conserved proteins found in members of the *Pasteurellaceae, Vibrionaceae*, *Rhizobiaceae* and *Enterobacteriaceae*. HugX is also distributed amongst the *Vibrionaceae*, *Rhizobiaceae* and *Enterobacteriaceae* but until now, was known to be present in only one member of the *Pasteurellaceae*, *P. multocida*.

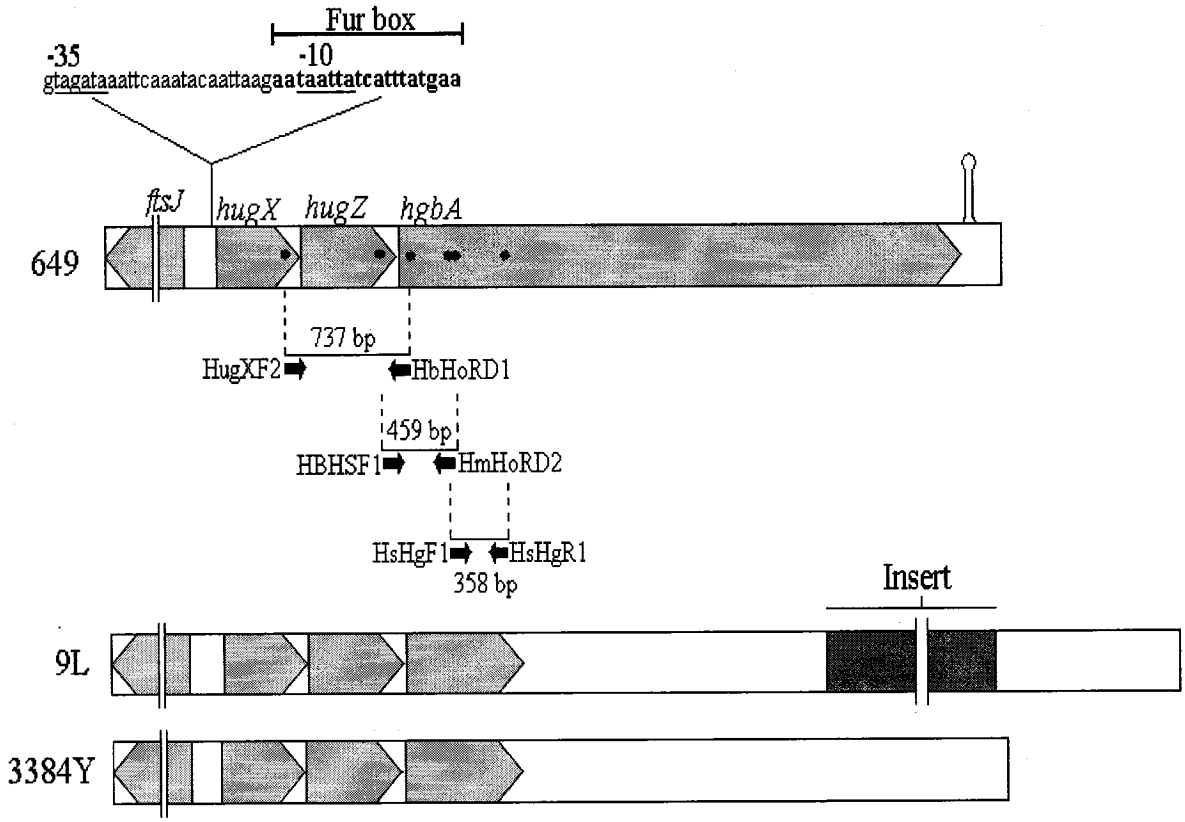


Fig. 3.13. Genetic organization of the *hugXZ-hgbA* operon in *H. somni* strains 649, 9L and 3384Y. All three genes are preceded by typical Shine-Dalgarno sequences. In strain 649, a stem loop is present after the stop codon of *hgbA* suggesting a Rho-independent mechanism for transcription terminator. An insert of undetermined length is present in strain 9L. The locations of the primers used in the initial PCR, in the phase variation assays and in RT-PCR are indicated and represented by black dots.

In strain 649, *hgbA* is predicted to encode a homologue of Hb-binding proteins found in other members of the *Pasteurellaceae* and in the pathogenic neisseriae. The closest matches were the Hb-binding proteins of *A. actinomycetemcomitans* (711/1131, 62%), *H. influenzae* (687/1115, 61%) and *A. pleuropneumoniae* (651/1135, 57%). In contrast, in strains 9L and 3384Y, HgbA would appear to be truncated due to a two base-pair deletion near the 5' ends of the structural genes. Interestingly, an unknown insert is also present in "*hgbA*" of strain 9L; more than 500 bp of this insert was sequenced but BLAST searches did not yield any significant match.

Typical Shine-Dalgarno sequences were identified upstream of *hugX*, *hugZ* and *hgbA*. Typical promoter sequences, however, and Fur boxes, were found upstream of only *hugX*. In strain 649, a stem loop was identified downstream of *hgbA* suggesting a Rho-independent mechanism for transcription termination. Putative cleavage sites, resulting in mature HgbA proteins, were identified using SignalP V3.0 (http://www.cbs.dtu.dk/services/SignalP/; Bendtsen *et al.*, 2004) and the molecular mass and pI of the predicted, mature HgbA of strain 649 were calculated (Compute pI/Mw; http://ca.expasy.org/tools/pi_tool.html) to be 124 kDa and 8.87, respectively. Motif and pattern searches and analyses also revealed the probable presence of a TonB-box and the highly-conserved amino acid motifs of Hm- and Hb-binding proteins, FRAP and NPNL ('NIQL' in HgbA).

The presence of putative promoter sequences and Fur boxes upstream of only *hugX* and the arrangement of *hugX*, *hugZ* and *hgbA* suggested that all three genes were transcribed as a single unit. RNA harvested from *H. somni* strain 649 grown under iron-replete and iron-restricted conditions was used in RT-PCR. A 737-bp *hugX-hgbA* fragment was detected when the RNA template was from cells grown under iron-

restricted conditions but not when the RNA was from cells grown under iron-replete conditions (Fig 3.14). These results suggest that *hugX*, *hugZ* and *hgbA* are arranged in an operon that it is up-regulated in response to iron-restriction.

3.5 Expression of HgbA can be subject to a form of phase variation involving poly C tracts within the structural genes

Based on previous experiences with *H. somni* (Ekins and Niven, 2003; Ekins *et al.*, 2004a), the apparent requirement for Hb in the growth medium for the expression of Hb-binding activity by strains 2336 and 8025, and the longer lag phases (with respect to strain 649) observed with these strains, suggested that in strains 2336 and 8025, HgbA may be subject to phase variation. Using the incomplete genome sequence of *H. somni* strain 2336, a region that appeared to encode an HgbA homologue was compared with *hgbA* of strain 649 and led to the identification of a poly C tract that might be responsible for strand-slippage and hence, phase variation. A 459-bp region containing the poly C tract was amplified by means of PCR and sequenced from all three strains (649, 2336 and 8025) grown under iron-replete conditions and under iron-restricted conditions in the presence of bovine Hb. Sequence analyses revealed that in strain 649, the poly C tract contained 6 C's irrespective of the growth conditions; in contrast, strains 2336 and 8025 possessed 8 and 10 C's, respectively, when grown under iron-replete conditions and 9 C's when grown under iron-restricted conditions in the presence of bovine Hb.

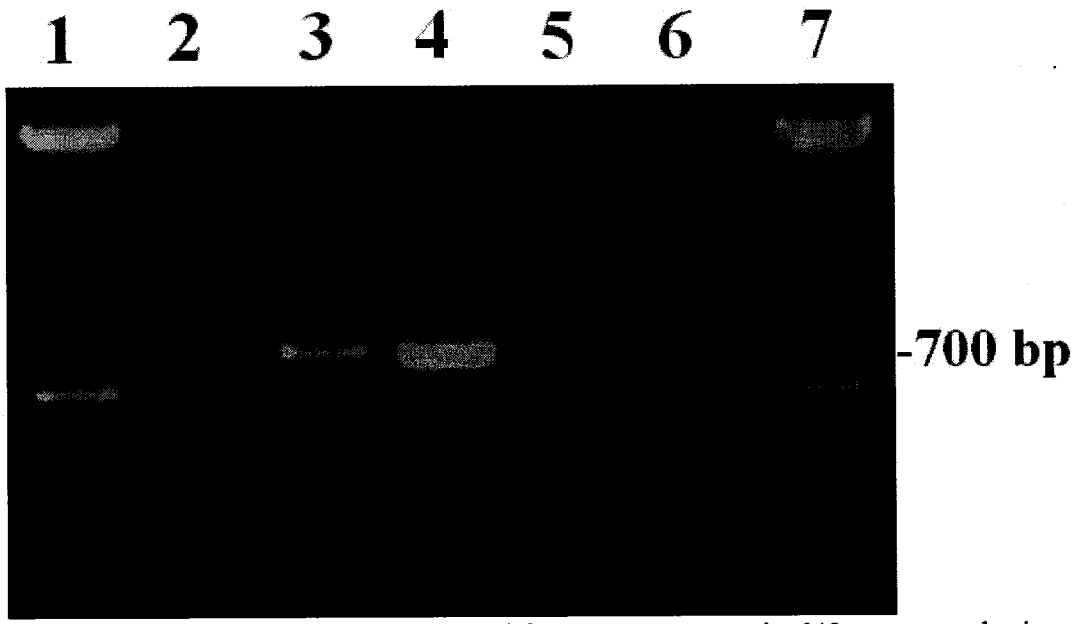


Fig. 3.14. RT-PCR using RNA isolated from *H. somni* strain 649 grown under iron-replete (lane 2) or iron-restricted (lane 3) conditions, or with total DNA (lane 4). Lanes 5 and 6 represent negative controls in which RNA from organisms grown under iron-replete (lane 5) and iron-restricted (lane 6) conditions served as templates in PCR. The primers were chosen to amplify a 737-bp *hugX-hgbA* fragment. Lanes 1 and 7 contained a 100-bp DNA ladder.

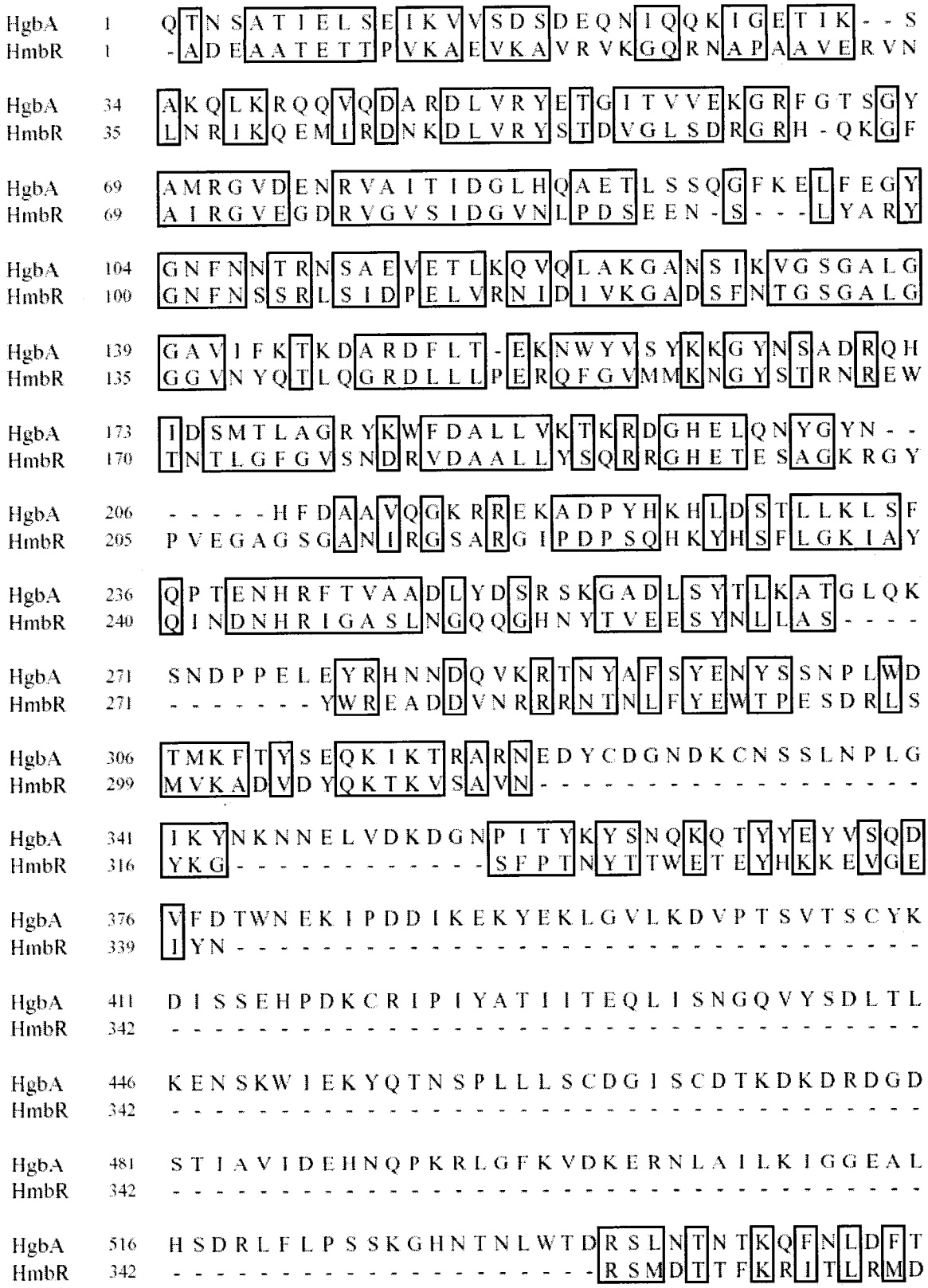
Chapter 4 - Discussion

The abilities of bovine (strains 649, 2336 and 8025) and ovine (strains 9L, 3384Y, 642A, 714 and 5688T) isolates of *H. somni* to utilize various Hbs and Hm as sources of iron were investigated. Strains 649, 2336 and 8025 were capable of utilizing iron from bovine Hb but not from ovine, porcine or human Hb or from Hm. Conversely, ovine isolates could not use any of the tested Hbs or Hm as an iron source for growth. The Hb and Hm solutions were tested with a strain of *A. suis* and all the Hb and Hm solutions supported the growth of *A. suis*. Also, increasing the concentrations of the tested Hbs and Hm had no noticeable impact on the growth rate of *H. somni* strain 649. The discrimination exhibited by *H. somni* is quite unusual and is the first reported case of such specificity in terms of utilization of Hb-bound iron. Lack of utilization of Hm as an iron source by *H. somni* is also in conflict with a previous report (Pontarollo *et al.*, 1997). The binding of biotinylated Hb by intact cells, however, did not display the same specificity. Bovine, ovine and human Hbs were bound by intact cells of strains 649 grown under iron-restricted conditions and by strains 649, 2336 and 8025 grown under iron-restricted conditions in the presence of bovine Hb. The binding activity was affected by trypsin in that bovine, porcine and human Hbs were not bound by treated cells; ovine Hb, however, was bound but with a diminished capacity. Native bovine, ovine and human Hbs could compete with biotinylated bovine Hb for the binding site(s) on the organisms but native porcine Hb could not. These observations suggest that bovine, ovine and human Hbs are bound by the same protein receptor(s). A binding mechanism involving the globulin moiety rather than the Hm moiety offers a possible explanation for the disparity between *H. somni* and other *Pasteurellaceae* with respect to the specificities of Hb binding. The

binding of ovine Hb by trypsin-treated cells suggests that ovine Hb may be bound by some other component (e.g., lipooligosaccharide) of the outer membrane.

The disparity between the utilization and binding profile of *H. somni* is puzzling. An explanation may lie in the 2D model of the meningococcal HmbR (Perkins-Balding *et al.*, 2003). As indicated in the model, some external loops are required for binding whereas other external loops are necessary for utilization of Hb-bound iron. HmbR has been shown to bind and to allow the acquisition of iron from mammalian Hbs without discrimination. The predicted mature HgbA was aligned with and compared to HmbR (Fig 4.1). The regions comprising the Hb-binding site show some significant similarities; however, the utilization sites show limited similarities with the exception of the region surrounding the FRAP and NPQL motifs.

SDS-PAGE analysis of the total and outer membranes of *H. somni* strains 649, 2336 and 8025 revealed the presence of an ~120-kDa polypeptide when the organisms were grown under the same growth conditions that promoted the expression of Hb-binding activity. Correlation between the results from the Hb-binding assays and the SDS-PAGE analyses suggests that the ~120-kDa polypeptide is the outer membrane protein responsible for the Hb-binding phenotype. Attempts to affinity-isolate Hb-binding proteins using the method developed by Elkins (1995) were not successful; in this protocol, the binding ligand (Hb) is cross-linked to beaded agarose and it may be that the agarose interfered with the binding of the Hb to the receptor(s). The methods of Ricard *et al.* (1991) and Jin *et al.* (1996), using biotinylated bovine Hb as the binding ligand, were also tried but without success. The effectiveness of the solubilizing solutions was also questioned. To address this possibility, the method of Elkins (1995) was modified such that the detergent solutions contained the critical micelle concentration of Zwittergent.


HgbA 1 QTNSATIELSEIKVVSDSDEQNIQQKIGETIK--S
HmbR 1 -ADEAATETTPVKAEVKAVRVKGQRNAPAAVERVN
HgbA 34 AKQLKRQQVQDARDLVRYETGITVVEKGRFGTSGY
HmbR 35 LNRIKQEMIRDNKDLVRYSTDVGLSDRGRH-QKGF
HgbA 69 AMRGVDENRVAITIDGLHQAETLSSQGFKELFEGY
HmbR 69 AIRGVEGDRVGVSIDGVNLPDSEEN-S---LYARY
HgbA 104 GNFNNTRNSAEVETLKQVQLAKGANSIKVGSGALG
HmbR 100 GNFNSSRLSIDPELVRNIDIVKGADSFNTGSGALG
HgbA 139 GAVIFKTKDARDFLT-EKNWYVSYKKGYNSADRQH
HmbR 135 GGVNYQTLQGRDLLLPERQFGVMMKNGYSTRNREW
HgbA 173 IDSMTLAGRYKWFDALLVKTKRDGHELQNYGYN--
HmbR 170 TNTLGFGVSNDRVDAALLYSQRRGHETESAGKRGY
HgbA 206 -----HFDAAVQGKRREKADPYHKHLDSTLLKLSF
HmbR 205 PVEGAGSGANIRGSARGIPDPSQHKYHSFLGKIAY
HgbA 236 QPTENHRFTVAADLYDSRSKGADLSYTLKATGLQK
HmbR 240 QINDNHRIGASLNGQQGHNYTVEESYNLLLAS----
HgbA 271 SNDPPELEYRHNNDQVKRTNYAFSYENYSSNPLWD
HmbR 271 -------YWREADDVNRRRNTNLFYEWTPESDRLS
HgbA 306 TMKFTYSEQKIKTRARNEDYCDGNDKCNSSLNPLG
HmbR 299 MVKADVDYQKTKVSAVN----------------
HgbA 341 IKYNKNNELVDKDGNPITYKYSNQKQTYYEYVSQD
HmbR 316 YKG----------SFPTNYTTWETEYHKKEVGE
HgbA 376 VFDTWNEKIPDDIKEKYEKLGVLKDVPTSVTSCYK
HmbR 339 IYN----------
HgbA 411 DISSEHPDKCRIPIYATIITEQLISNGQVYSDLTL
HmbR 342 ----------
HgbA 446 KENSKWIEKYQTNSPLLLSCDGISCDTKDKDRDGD
HmbR 342 ----------
HgbA 481 STIAVIDEHNQPKRLGFKVDKERNLAILKIGGEAL
HmbR 342 ----------
HgbA 516 HSDRLFLPSSKGHNTNLWTDRSLNTNTKQFNLDFT
HmbR 342 ----------RSMDTTFKRITLRMD

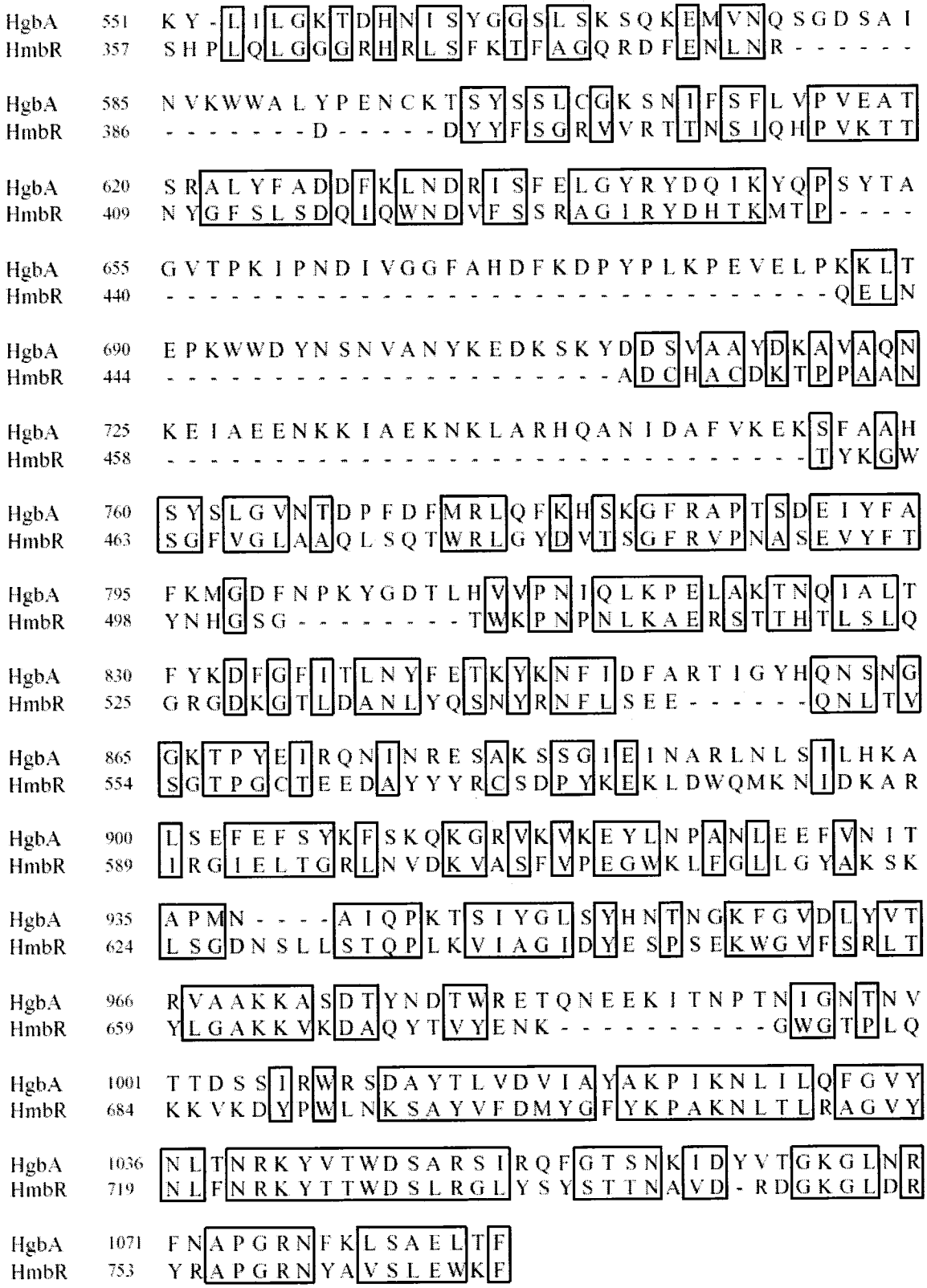

HgbA 551 KY-LILGKTDHNISYGGSLSKSQKEMVNQSGDSAI
HmbR 357 SHPLQLGGGRHRLSFKTFAGQRDFENLNR------
HgbA 585 NVKWWALYPENCKTSYSSLCGKSNIFSFLVPVEAT
HmbR 386 -------D-----DYYFSGRVVRTTNSIQHPVKTT
HgbA 620 SRALYFADDFKLNDRISFELGYRYDQIKYQPSYTA
HmbR 409 NYGFSLSDQIQWNDVFSSRAGIRYDHTKMTP----
HgbA 655 GVTPKIPNDIVGGFAHDFKDPYPLKPEVELPKKLT
HmbR 440 ----------------------------QELN
HgbA 690 EPKWWDYNSNVANYKEDKSKYDDSVAAYDKAVAQN
HmbR 444 ----------------------ADCHACDKTPPAAN
HgbA 725 KEIAEENKKIAEKNKLARHQANIDAFVKEKSFAAH
HmbR 458 ----------------------------TYKGW
HgbA 760 SYSLGVNTDPFDFMRLQFKHSKGFRAPTSDEIYFA
HmbR 463 SGFVGLAAQLSQTWRLGYDVTSGFRVPNASEVYFT
HgbA 795 FKMGDFNPKYGDTLHVVPNIQLKPELAKTNQIALT
HmbR 498 YNHGSG---------TWKPNPNLKAERSTTHTLSLQ
HgbA 830 FYKDFGFITLNYFETKYKNFIDFARTIGYHQNSNG
HmbR 525 GRGDKGTLDANLYQSNYRNFLSEE------QNLTV
HgbA 865 GKTPYEIRQNINRESAKSSGIEINARLNLSILHKA
HmbR 554 SGTPGCTEEDAYYYRCSDPYKEKLDWQMKNIDKAR
HgbA 900 LSEFEFSYKFSKQKGRVKVKEYLNPANLEEFVNIT
HmbR 589 IRGIELTGRLNVDKVASFVPEGWKLFGLLGYAKSK
HgbA 935 APMN----AIQPKTSIYGLSYHNTNGKFGVDLYVT
HmbR 624 LSGDNSLLSTQPLKVIAGIDYESPSEKWGVFSRLT
HgbA 966 RVAAKKASDTYNDTWRETQNEEKITNPTNIGNTNV
HmbR 659 YLGAKKVKDAQYTVYENK----------GWGTPLQ
HgbA 1001 TTDSSIRWRSDAYTLVDVIAYAKPIKNLILQFGVY
HmbR 684 KKVKDYPWLNKSAYVFDMYGFYKPAKNLTLRAGVY
HgbA 1036 NLTNRKYVTWDSARSIRQFGTSNKIDYVTGKGLNR
HmbR 719 NLFNRKYTTWDSLRGLYSYSTTNAVD-RDGKGLDR
HgbA 1071 FNAPGRNFKLSAELTF
HmbR 753 YRAPGRNYAVSLEWKF

Fig 4.1. Sequence alignment of the mature HgbA of *H. somni* strain 649 (HgbA) and the mature HmbR of *N. meningitidis* (HmbR). The extracellular loop responsible for binding Hb, L2 and L3, are located between the amino acid residue (aa) 192 and 230, and aa 254 and 284 of the HmbR sequence, respectively. Loop L6, L7 and L10, which plays a role in acquisition of iron from Hb, are located between the aa 420 and 462, aa 486 and 516, and aa 660 and 698 of the HmbR sequence, respectively. The FRAP and NIQL motifs are located between aa 487 and 490, and 509 and 512 of the HmbR sequence, respectively.

This modification, however, did not allow the affinity-isolation of the protein(s) of interest.

hugX and *hugZ* homologues and a Hb-binding protein gene (*hgbA*) homologue were identified using a PCR approach. RT-PCR experiments demonstrated that all three genes are arranged in operon as in *P. multocida* (Bosch *et al.*, 2002). This operon was observed to be iron-repressible and the regulatory region upstream of *hugX* was found to possess a Fur box. Binding of Fur to the regulatory region of the *hugXZ-hgbA* operon has been demonstrated by FURTA assay and electrophoretic mobility shift assays (G. Bonfils, Y.D.N. Tremblay and D.F. Niven, unpublished data). The predicted protein encoded by *hgbA* is a homologue of other proteins belonging to a family of TonB-dependent, outer-membrane, Hb-binding proteins. The pI of HgbA was predicted to be 8.87, which is within the range of most outer-membrane proteins. A predicted TonB box was also identified in the C-terminal region of HgbA. All of the above, predicted features are characteristic of Hb-binding proteins. The predicted, mature HgbA has a deduced molecular mass of 124 kDa suggesting that the iron-regulated 120-kDa outer membrane polypeptide observed in the SDS-PAGE analyses is HgbA. Furthermore, ovine strains failed to bind, and to acquire iron from, Hb and are predicted to produce a truncated HgbA. Hence, HgbA may well be the only component responsible for Hb-binding activity and the acquisition of iron from Hb.

A poly C tract within the structural gene for HgbA was predicted to be involved in phase variation in the bovine strains of *H. somni*. When grown under iron-replete conditions, strains 2336 and 8025 were shown to possess poly C tracts with 8 and 10 C's, respectively. In contrast, when grown under iron-restricted conditiona in the presence of Hb, the poly C tracts in both strains (2336 and 8025) were demonstrated to have 9 C's.

Lewis *et al.* (1999) demonstrated that a change in the number of G's in the poly G tracts in the Hb-binding-protein genes of *N. meningitidis* influenced the Hb-binding phenotype in that the abilities of the organism to acquire iron from Hb and to produce Hb-binding proteins were modified. Similar results were observed with the Hb-binding-protein genes of *H. influenzae* (Cope *et al.*, 2000). Such changes in phenotype suggest that growth in the presence of Hb selects for cells that possess a functional Hb-binding protein. In addition, phase variation in the Tf-binding-protein genes of *H. somni* has been shown to be responsible for changes in the expression of Tf-binding activity and in the production of Tf-binding proteins (Ekins and Niven, 2003; Ekins *et al.*, 2004). In strains 2336 and 8025, phase variation of *hgbA* correlates with the longer growth cycle (with respect to strain 649), the lack of Hb-binding activity and the absence of the 120-kDa outer-membrane polypeptide in organisms grown under iron-restricted conditions. These observations, once again, suggest strongly that the ~120-kDa polypeptide is HgbA and is required for expression of the Hb binding phenotype.

The role of HgbA as a virulence factor is questionable. Although all strains tested in this study were isolated from diseased animals, only the bovine isolates utilized Hb-bound iron. Haemolysis of erythrocytes is an important step in the process of iron acquisition from Hb and in virulence. Notably, ovine isolates cannot damage erythrocytes and with most bovine isolates, haemolysis was observed with bovine erythrocytes but not with ovine erythrocytes (Stephens *et al.*, 1983; Webb, 1983a; Ward *et al.*, 1995). Two strains tested, strains 649 and 8025, are haemolytic (Ward *et al.*, 1995) and in these strains, HgbA may play a limited role in virulence. On the other hand, a number of non-haemolytic, pathogenic strains, such as strain 2336, can still utilize Hb as an iron source suggesting, once again, that HgbA is not a critical virulence factor for *H. somni*.

Although HugX and HugZ homologues have been identified and partially characterized in *P. multocida* (Bosch *et al.*, 2002) and *P. shigelloides* (Henderson *et al.*, 2001), the exact roles of HugX and HugZ are unknown. Both proteins are required for the utilization of Hb and Hm as iron sources as demonstrated with knockout mutants. It has been suggested that HugX and HugZ may prevent Hm toxicity. BLASTP searches revealed that HugX had significant similarities with the C-terminal regions of the probable coproporphyrinogen III oxidases of *H. ducreyi* (GenBank Accession no. NP 872663) and *A. pleuropneumoniae* (GenBank Accession no. ZP 00135019). Coproporphyrinogen III oxidase plays a role in the synthesis of Hm and the crystal structure of HemN, a coproporphyrinogen III oxidase, from *E. coli* has been established (Layer *et al.*, 2003). The C-terminal region of HemN appears to bind the substrate, coproporphyrinogen III. Although the C-terminal of HemN of *E. coli* and *H. ducreyi* share few similarities, the data suggest, to a certain extent, that HugX may bind Hm. Analysis of the C-terminal region of coproporphyrinogen III oxidase of *H. ducreyi* may help in the determination of the function of HugX.

Motif, domain and pattern searches did not reveal any significant matches for HugX; however, HugZ possesses a domain found in the pyridoxamine 5'-phosphate oxidase family. These enzymes play a role in the *de novo* synthesis of pyridoxine (vitamin B6) and pyridoxal phosphate by oxidizing pyridoxamine-5-phosphate and pyridoxine-5-phosphate to pyridoxal-5-phosphate. HugZ might be a Hm oxidase, oxidoreductase or oxygenase and the role of HugZ may be the removal of iron from Hm. It is tempting to suggest that HugX binds Hm as it enters the cytoplasm, thereby limiting the toxic action of Hm. HugZ then removes the iron by either degrading Hm or catalysing a reduction reaction. Together, HugX and HugZ might possess some functions similar to

those of HemO of *N. meningitidis* (Zhu *et al.*, 2000). HugZ is widely distributed amongst members of *Pasteurellaceae* and since HugX, to date, has been shown to be present in only two members, *H. somni* and *P. multocida*, it is possible that HugZ works independently in the other members of *Pasteurellaceae*, or perhaps, in cooperation with functional homologues of HugX.

A poly C tract within the structural gene of HgbA is responsible for the phase variation observed in strains 2336 and 8025. Phase variation has been observed previously in *H. somni* (Ekins and Niven, 2003; Ekins *et al.*, 2004a) and amongst the identified Hb-binding-protein genes (Chen *et al.*, 1999; Lewis *et al.*, 1999; Ren *et al.*, 1999). It has been suggested that phase variation may be advantageous for bacteria in various ways such as immune system evasion and rapid host environment adaptation (Richardson and Stojiljkovic, 2001; van der Woude and Bäumler, 2004). For *H. somni*, the exact role of phase variation of HgbA is puzzling. The natural habitat of *H. somni* has been suggested to be the genital and respiratory tracts. Since ruminants do not menstruate, the presence of Hb in the genital tract is probably sporadic and rare. Therefore, phase variation might help *H. somni* to adapt rapidly in response to the available sources of iron in the host. Jerse *et al.* (2002) have shown that *N. gonorrhoeae* does not require Hb for growth in the genital tract of the female mouse. In the same study, the presence of blood also correlated with an increase in the frequency of phase variants with a functional Hb receptor. For example, cells of haemolytic strains, such as strain 8025, possessing a phase "on" HgbA would have an advantage over phase "off" variants when erythrocytes are present in the genital and respiratory tracts or once the organisms have entered the blood stream. Phase "on" variants would be able to acquire iron from Hb from lysed erythrocytes, enhancing the infection process.

It is concluded that *H. somni* possesses a system for the acquisition of Hb-bound iron analogous to those found in other *Pasteurellaceae* and that the system is subjected to phase variation in strains 2336 and 8025. However, the specificities of the Hb receptors are quite unusual. It is also suggested that for *H. somni,* Hb is probably a minor source of iron in the host and other sources such as Tf and siderophores produced by the mucosal flora are likely to be major sources of iron. In addition, it is unlikely that HgbA contributes significantly to the virulence of *H. somni*.

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