Iron acquisition by *Histophilus ovis*

by

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"Chance favours the prepared mind"

-Louis Pasteur

Abstract

Ph. D. **Andrew John Ekins** **Microbiology**

Iron acquisition by *Histophilus ovis*

Five strains (9L, 642A, 714, 5688T and 3384Y) of *Histophilus ovis* were investigated with respect to iron acquisition. All strains used ovine, bovine and goat, but not porcine or human, transferrins (Tfs) as iron sources for growth. In solid phase binding assays, total membranes from only two (9L and 642A) of the five strains, grown under iron-restricted conditions, were able to bind Tfs (ovine, bovine and goat, but not porcine or human). However, when the organisms were grown under iron-restricted conditions in the presence of bovine Tf, total membranes from all strains exhibited Tf binding (as above); competition experiments demonstrated that all three Tfs (ovine, bovine and goat) were bound by the same receptor(s). An affinity isolation procedure allowed the isolation of two putative Tf-binding polypeptides (78 and 66 kDa) from total membranes of strains 9L and 642A grown under iron-restricted conditions, and from membranes of all strains if the growth medium also contained Tf. A gene encoding a *Pasteurella multocida* TbpA homologue was shown to be present in each of two representative strains (9L and 3384Y); these genes were sequenced and determined to be the structural genes encoding the 78-kDa Tf-binding polypeptides. The identification of a *fur* homologue and a Fur box within the promoter region of *tbpA* in both strains indicated that Fur (and iron) is responsible for the iron-repressible nature of Tf-binding activity. Although *tbpA* transcripts were detected by reverse transcription

(RT)-PCR with RNA isolated from strains 9L and 3384Y grown under iron-restricted conditions, with strain 3384Y, and depending on the primer pair, *tbpA* transcripts were detected by RT-PCR predominantly when the RNA was isolated from cells grown under conditions of iron-restriction in the presence of Tf. The presence of an additional G in the *tbpA* gene of strain 3384Y grown under iron-replete conditions, compared to organisms grown under iron-restricted conditions plus bovine Tf, is believed to be responsible for the Tf-dependent expression of Tf-binding activity by strain 3384Y (and possibly 5688T and 714) by causing a frame shift that results in the introduction of a premature stop codon.

Résumé

Ph. D. **Andrew John Ekins** **Microbiologie**

Acquisition du fer par *Histophilus ovis*

L'acquisition de fer par cinq souches (9L, 642A, 714, 5688T et 3384Y) d'*Histophilus ovis* a été analysée. Toutes les souches étaient capables d'utiliser les transferrines (Tfs) d'origine ovine, bovine et caprine comme source de fer pour leur croissance mais pas les Tfs porcines et humaines. Après des tests de liaison en phase solide, les membranes totales de seulement deux souches (9L et 642A) sur cinq cultivées dans des conditions restreintes en fer, étaient capables de lier la Tf (ovine, bovine et caprine, mais pas porcine et humaine). Cependant, lorsque cultivées dans les mêmes conditions en présence de Tf d'origine bovine, les membranes totales de toutes les souches sans exception se sont montrées capables de lier la Tf (comme précédemment). Des tests de compétition ont démontré que les trois Tfs (ovine, bovine et caprine) étaient liées par le(s) même(s) récepteur(s). Une technique d'isolement par affinité a permis l'identification de deux polypeptides putatifs de 78 et 66 kDa pouvant lier la Tf à partir des membranes totales des souches 9L et 642A cultivées dans des conditions restreintes en fer, et à partir des membranes totales de toutes les souches à condition que le milieu contienne aussi de la Tf. Un gène codant pour un homologue de la protéine TbpA de *Pasteurella multocida* a été identifié chez chacune des deux souches représentatives (9L et 3384Y); le séquençage de ce gène confirme qu'il code

pour un polypeptide putatif de 78 kDa pouvant lier la Tf. L'identification d'un homologue de *fur* ainsi que d'une boîte Fur dans la région du promoteur de *tbpA* chez les deux souches suggère que Fur (et le fer) est responsable de la régulation (par le fer) de sa propriété de liaison à la Tf. Bien que les produits de transcription de *tbpA* soient détectés par transcription inverse (RT)-PCR avec l'ARN isolé des souches 9L et 3384Y cultivées dans des conditions restreintes en fer, pour la souche 3384Y, selon la paire d'amorces utilisée, les produits de transcription de *tbpA* sont détectés en majorité par RT-PCR surtout lorsque l'ARN provenait des cellules cultivées dans les conditions restreintes en fer en présence de Tf. La présence d'un G additionnel dans la séquence du gène *tbpA* de la souche 3384Y cultivée dans des conditions optimales en fer en comparaison à la même souche cultivée dans des conditions restreintes en fer en présence de Tf bovine, est soupçonnée d'être responsable de l'expression Tf-dépendante de TbpA par la souche 3384Y (et probablement aussi 5688T et 714) en causant un changement du cadre de lecture qui amènerait l'introduction prématurée d'un codon de terminaison.

Contributions to knowledge

1. *Histophilus ovis* strains 9L, 642A, 3384Y, 5688T and 714 can use ovine, bovine and goat Tfs, but not human or porcine Tfs, as iron sources for growth.

2. Two strains (9L and 642A) of *H. ovis* are capable of binding ovine, bovine and goat Tfs, but not human or porcine Tfs when grown under iron-restricted conditions in the presence or absence of a Tf that supports growth.

3. Three strains (3384Y, 5688T and 714) of *H. ovis* are capable of binding ovine, bovine and goat Tfs, but not human or porcine Tfs only when grown under iron-restricted conditions in the presence of a Tf that supports growth.

4. Competition experiments demonstrated that all three ruminant Tfs are bound by a common receptor.

5. *H. ovis* produces Tf-binding polypeptides of 78 and 66 kDa under the same conditions that induce Tf-binding activity.

6. Two representative strains (9L and 3384Y) of *H. ovis* possess a *tbpA* homologue that encodes the 78-kDa Tf-binding polypeptide.

7. The promoters of the *tbpA* homologues of both strains are identical and contain a putative Fur box.

8. The predicted *H. ovis* TbpA proteins are homologues of the *Pasteurella multocida* TbpA protein and collectively represent the second example of a new subfamily of TonB-dependent receptors.

9. Two representative strains (9L and 3384Y) of *H. ovis* possess *fur* and *fldA* homologues.

10. *fldA* is 16 bp upstream of *fur* and the two genes are transcribed as an operon.

11. The relative amounts of the *fldA-fur* transcripts are not affected by the availability of iron or the presence or absence of Tf in the growth medium.

12. *tbpA*-specific transcripts are detected in two strains (9L and 3384Y) when the organisms are grown under iron-restricted conditions.

13. Increased amounts of *tbpA*-specific transcripts are detected in strain 3384Y when the organisms are grown under iron-restricted conditions in the presence of Tf.

14. An additional G is present in the *H. ovis* 3384Y *tbpA* gene when the organism is grown under iron-replete conditions compared to when the organism is grown under iron-restricted conditions in the presence of Tf.

15. The additional G present in the *H. ovis* 3384Y *tbpA* gene is predicted to be responsible for the Tf-dependent expression of TbpA.

List of abbreviations

EDDA	Ethylenediamine di-*o*-hydroxyphenylacetic acid
FURTA	Fur titration assay
HRP	Horseradish peroxidase
Lf	Lactoferrin
PVDF	Polyvinylidene difluoride
RT-PCR	Reverse transcription-PCR
SOD	Superoxide dismutase
sTYE-H	Supplemented TYE-H
TBS	Tris-buffered saline
Tf	Transferrin
TTBS	TBS containing Tween 20
TY	Tryptone-yeast extract medium
TYE-H	HEPES-buffered tryptone-yeast extract medium

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Contributions of authors

All of the experiments described in this thesis were performed by myself, under the supervision of Dr. D.F. Niven. Manuscripts for publication were originally written by myself and such manuscripts were subsequently edited and revised based on the comments of Dr. Niven. Revisions for publication were done by both Dr. Niven and myself.

Chapter 1. Literature review

1.1 Introduction

In a recent review of iron metabolism in pathogenic bacteria (Ratledge and Dover, 2000), the authors speculated that the number of publications dealing with microbial iron metabolism in the ten years preceding their review "exceeds the total number of all previous publications in the field" while the authors of a 78-page chapter on bacterial iron transport (Braun *et al.*, 1998) presented only certain iron transport systems since "to cover all that is known on bacterial iron transport would in itself fill a book". I am inclined to agree with these two statements and in an effort to concisely set the scene for this thesis, this literature review will focus primarily on recent advances in the study of iron acquisition strategies employed by Gram-negative bacteria, with an obvious bias towards systems used by pathogenic members of the families *Pasteurellaceae* and *Neisseriaceae*.

1.2 *Histophilus ovis*

Histophilus ovis, a member of the *Pasteurellaceae*, is a Gram-negative pleomorphic rod, initially isolated and described by Roberts (1956), capable of causing a variety of disease symptoms in sheep including epididymitis in rams, abortion in ewes, and synovitis and septicaemia in lambs (Rahaley and White, 1977; Rahaley, 1978a; Rahaley, 1978b; Webb, 1983); more recently, it has also been implicated in cases of thrombotic meningoencephalitis (Cassidy *et al.*, 1997). DNA hybridization (Walker *et al.*, 1985) and biochemical (Stephens *et al.*, 1983) data indicate that *H. ovis*

is closely related to both *Haemophilus somnus* and *Haemophilus agni* and many believe that all three organisms belong to a single species (Stephens *et al.*, 1983; Walker *et al.*, 1985; Piechulla *et al.*, 1986; Kirkham *et al.*, 1989) with the ovine and bovine isolates representing two distinct groups within this species (Walker *et al.*, 1985; Kirkham *et al.*, 1989). Despite these beliefs, organisms possessing characteristics of this group and isolated in Australia are referred to commonly as *H. ovis* (McGillivery *et al.*, 1986), regardless of the isolates being of ovine or bovine origins, while organisms isolated in North America are referred to commonly as *H. somnus* (Ward *et al.*, 1995). Interestingly, *H. ovis* and *H. somnus* can be differentiated using restriction enzyme analysis of chromosomal DNA (McGillivery *et al.*, 1986; Kirkham *et al.*, 1989) or by a PCR-ribotyping method (Appuhamy *et al.*, 1998).

1.3 Iron

1.3.1 Importance of iron

Although iron is the fourth most abundant element within the Earth's crust (Crichton and Pierre, 2001), under aerobic conditions and at neutral pH, the concentration of freely available iron (as Fe^{3+}) in solution is estimated to be somewhere between 10^{-12} and 10^{-9} M (Braun *et al.*, 1998; Chipperfield and Ratledge, 2000). Iron requiring pathogens of vertebrates encounter an even lower concentration of free iron, of approximately 10^{-18} M, within the fluids of their hosts due to the iron sequestering activities of the iron-binding proteins transferrin (Tf) and lactoferrin (Lf) (Griffiths, 1987). This concentration of iron is well below that of approximately 10^{-7} M required for the growth of most bacterial species (Griffiths, 1987).

Iron can exist predominantly in two states of oxidation, namely the ferrous (Fe^{2+}) or the ferric (Fe^{3+}) states, possessing redox potentials ranging from approximately –300 to +700 mV, depending on the environmental conditions and associated ligands (Aisen *et al.*, 2001; Braun *et al.*, 1998). Due to this versatility, iron is used commonly as a cofactor in many enzymes (e.g. aconitase and catalase) and components of the electron transport chain (e.g. cytochromes) (Braun and Killman, 1999; Byers and Arceneaux, 1998; Crichton and Pierre, 2001). Consequently, with the exception of a few bacterial species (Archibald, 1983; Imbert and Blondeau, 1998; Niven and Ekins, 2001; Niven *et al.*, 1999; Posey and Gherardini, 2000), nearly all forms of life on earth have an absolute requirement for iron (Aisen *et al.*, 2001).

1.3.2 Iron and oxidative stress

Although iron can participate in a variety of reactions that are beneficial and/or essential to the survival of a bacterial cell, the reactive nature of iron can also lead to the formation of undesirable end products. The reactive oxygen species, superoxide (O_2^-) and hydrogen peroxide (H_2O_2), are unavoidable by-products of aerobic metabolism and are believed to arise principally from the activities of NADH dehydrogenase II, at least in *Escherichia coli* (Messner and Imaly, 1999). O_2^- is believed to exert its negative effect on bacterial cells by attacking enzymes containing [4Fe-4S] and releasing the iron to participate in the Fenton reaction (Gort and Imlay, 1998; Keyer *et al.*, 1995). Hydroxyl radical (OH•), produced as a result of the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow OH\bullet + Fe^{3+} + HO^-$; see e.g. Pierre and Fontecave, 1999), is capable of attacking and damaging a variety of biological molecules, including DNA (Keyer *et al.*, 1995). The

concentrations of O_2^- and H_2O_2 are normally maintained at tolerable levels within the bacterial cell by the activities of superoxide dismutase (SOD) and catalase enzymes, respectively (Byers and Arceneaux, 1998; Gort and Imlay, 1998), while the concentration of free iron is maintained at a suitable level through regulation of its import into the cell (see e.g Braun *et al*, 1998; Touati, 2000).

1.4 Regulation of iron uptake

1.4.1 Ferric uptake regulation (Fur)

Hantke (1981) first described a mutant of *E. coli* that constitutively expressed a number of genes involved in iron uptake, and named the mutant *fur* (for ferric uptake regulation). The gene was later cloned (Hantke, 1984) and sequenced (Schaffer *et al.*, 1985) revealing that Fur is a 17-kDa protein. Fur proteins form dimers through the interaction of their C-terminal domains (Stojiljkovic and Hantke, 1995) and exhibit their regulatory effect, when associated with iron (as Fe^{2+}), by binding to specific sequences of DNA within the promoter region of iron-regulated genes, blocking the binding of RNA polymerase and thereby inhibiting transcription of the gene (Escolar *et al.*, 1999). The N-terminal domains of dimerized Fur are responsible for DNA binding (Stojiljkovic and Hantke, 1995) and recognize the consensus sequence, 5' GATAATGAT **A** ATCATTATC 3', that can be viewed as two 9-bp inverted repeats separated by 1 bp (in bold), or alternatively, described as an array of the 6-bp sequence NAT(A/T)AT, repeated at least three times (Escolar *et al.*, 1998). The iron-uptake systems of nearly all Gram-negative bacteria examined to date are regulated either directly or indirectly by the (Fur) protein (Braun *et al.*, 1998). Fur is therefore a key

element involved in the maintenance of iron homeostasis within the cell and Fur (*fur*) homologues have been identified and described in a large number of bacterial species (see e.g. Escolar *et al.*, 1999; Touati, 2000).

1.4.2 *fur*: genetic organization and regulation

As the number of *fur* homologues sequenced increases, a picture of the genetic organization of *fur* is beginning to emerge. It would appear that the *fur* homologue within members of the β- and γ-proteobacteria is often preceded by either *omlA* or *smpA*, which encode homologous outer-membrane lipoproteins (Lowe *et al.*, 2001, and references therein); however, the *fur* homologues of certain γ-proteobacteria, including *Actinobacillus actinomycetemcomitans*, *E. coli*, *Klebsiella pneumoniae*, *Haemophilus influenzae*, *Pasteurella multocida*, and *Yersinia pestis*, are preceded by *fldA* which encodes a flavodoxin homologue (Achenbach and Genova, 1997; Haraszthy *et al.*, 2002; May *et al.*, 2001; Zheng *et al.*, 1999). It is speculated that flavodoxin may play a role in the response to oxidative stress by maintaining the reduced state of enzymatic [4Fe-4S] clusters, thereby protecting them from superoxide attack (Zheng *et al.*, 1999). It would appear that *fldA* is an essential gene in *E. coli* since mutants lacking *fldA* are not viable under either aerobic or anaerobic conditions; *fldA* insertion mutants could only be generated in strains of *E. coli* carrying an additional copy of *fldA* (Gaudu and Weiss, 2000). The close genetic association of *fldA-fur* is reasonable, as both are involved in the response to oxidative stress (Zheng *et al.*, 1999). Originally, it was thought that *fur* expression in *E. coli* was autoregulated (de Lorenzo *et al.*, 1988), as is possibly the case for *K. pneumoniae* (Achenbach and Yang, 1997) and the

cyanobacterium *Anabaena* PCC 7119 (Bes *et al.*, 2001), but it is now clear that the regulation of *fur* expression in *E. coli* is much more complex; the regulators of the response to oxidative stress belong to the OxyR and SoxRS systems and are capable of increasing the transcription of *fur* (Zheng *et al.*, 1999). Although Fur is capable of regulating the import of iron into the cell, maintaining the levels of free iron within the cells at a tolerable level and avoiding the production of OH•, it is also responsible for the activation or repression of a number of genes whose products play a direct role in the prevention of oxidative stress. *E. coli* possesses two cytosolic SODs, one Fe- and one Mn-co-factored, which are differentially expressed in response to the iron concentration, as perceived by Fur (Escolar *et al.*, 1999). The MnSOD is repressed by Fur in the classical sense (Touati, 2000) while expression of the FeSOD is activated by Fur by a post-transriptional mechanism involving transcript stabilization (Dubrac and Touati, 2000, 2002) by an antisense RNA, RyhB, and an RNA-binding protein, Hfq (Massé and Gottesman, 2002). This situation is logical since cells growing aerobically in an environment low in iron must still be able to cope with O_2^- and therefore express the MnSOD that does not compete for iron. When there is a higher concentration of iron, the FeSOD can be expressed to eliminate the generated O_2^- while taking up free iron and preventing it from participating in the Fenton reaction. Furthermore, the protein responsible for intracellular iron storage, ferritin, which can accommodate up to 4500 iron atoms, is activated by Fur by an unknown mechanism (Abdul-Tehrani *et al.*, 1999; Bereswill, *et al.*, 2000) that may involve the antisense RNA, RyhB, responsible for FeSOD activation (Massé and Gottesman, 2002). Hence, it is not surprising that *fur* mutants of some bacterial species, including *Neisseria meningitidis* (Thomas and

Sparling, 1994), *Pseudomonas aeruginosa* (Prince *et al.*, 1993), and *Rhizobium leguminosarum* (de Luca *et al.*, 1998) are not viable suggesting that Fur plays an essential role in these bacteria.

1.4.3 The Fur regulon

Aside from the involvement of Fur in protecting the cell against oxidative stress, due to the inherent reactivity of iron, Fur is also involved in the regulation of a variety of other cellular functions whose link to the iron status of the cell may or may not seem obvious. An imaginative and ingenious technique for identifying genes regulated by Fur, which takes advantage of the affinity of the Fur protein for the DNA sequence it binds (Fur box or iron box), was developed by Stojiljkovic *et al.* (1994). The system employed a reporter strain of *E. coli*, H1717 (Hantke, 1987), which has a promoterless *lacZ* gene fused to a Fur-regulated promoter. If a mid- to high-copy number plasmid, whose insert contains the Fur box sequence (or a close match), is introduced into H1717, the Fur proteins within the cell will bind to the Fur box inserted in the plasmid allowing expression of the chromosomal *lacZ* (Stojiljkovic *et al.*, 1994). Genes encoding iron-binding proteins will also allow the expression of the chromosomal *lacZ*, if the genes are expressed and functional within *E. coli*, as Fur cannot act as a repressor in the absence of iron. Using this technique, many unique Fur-regulated genes have been identified in *E. coli* (Sojiljkovic *et al.*, 1994) and *Salmonella typhimurium* (Tsolis *et al.*, 1995); more recently, the availability of complete genomic DNA sequences of a variety of bacterial species has allowed the use of computer-based searches for Fur boxes (Panina *et al.*, 2001) or DNA microarrays for the evaluation of gene expression

under iron-limiting conditions (Paustian *et al.*, 2001) for the identification of genes that are putative members of the Fur regulon.

Within pathogenic bacteria, an environment low in iron can signal the entry into the host (Mekalanos, 1992) and the production of a number of virulence determinants such as the Shiga-like toxin (e.g. Dirita and Mekalanos, 1989; Mekalanos, 1992) and urease (Heimer *et al.*, 2002) of *E. coli*, are regulated either directly or indirectly by Fur. Fur-dependent regulation of other genes, including *ompH*, which encodes the major outer membrane protein in *P. multocida*, remains enigmatic (Bosch *et al.*, 2001). The majority of Fur-regulated genes, however, are those involved in the acquisition of iron, or regulators of genes whose products are involved in iron acquisition (e.g., Braun *et al.*, 1998; Crosa, 1997).

1.5 Iron acquisition by bacterial pathogens

1.5.1 Bacterial iron requirements

The environment encountered by pathogens within their host is one of extreme iron-restriction (Griffiths, 1987). Therefore, pathogens must either lack a requirement for iron or have a mechanism for acquiring it within their host. Although the list is not long, there are some bacteria, including pathogens, which do not appear to require iron. The lactobacilli appear to not have an iron requirement (Archibald, 1983; Imbert and Blondeau, 1998) and are capable of excluding it (Archibald, 1983), a trait shared by the bacterium responsible for Lyme disease, *Borrelia burgdorferi* (Posey and Gheradini, 2000). *Streptococcus suis*, a pathogen of swine and humans, appears to require little, if any, iron (Niven *et al.*, 1999), but is incapable of excluding it (Niven and Ekins, 2001)

and as revealed by the complete genome sequence, the pathogen responsible for syphilis, *Treponema pallidum*, does not appear to have any iron-containing proteins (Posey *et al.*, 1999). The majority of pathogens, however, do require iron and they have evolved a number of mechanisms for acquiring it within their host.

1.5.2 Sources of iron within the host

Although there are ample supplies of iron within the host, the metal is not freely available to invading pathogens. Intracellular iron is found within the iron storage protein, ferritin, or complexed with haem and haemoproteins (Mietzner and Morse, 1994) and in order to gain access to such iron-containing complexes, invading pathogens must first be able to cause tissue destruction to allow their release (Mietzner and Morse, 1994). In the serum, free haemoglobin is bound quickly by haptoglobin and haem is bound by either haemopexin or albumin, and these complexes are removed from the circulation by the liver (Mietzner and Morse, 1994; Wandersman and Stojiljkovic, 2000). Iron in the fluids of vertebrates is bound to the proteins of the transferrin family, Tf and Lf, which are found primarily in serum and mucosal secretions, respectively (Aisen, 1998; Griffiths, 1987). Lf is structurally similar to Tf but is able to bind iron under more acidic conditions (pH 4.5) and is thought to exhibit antimicrobial activities, possibly by the generation of hydroxyl radical (OH•), catalyzed by the bound iron (Mietzner and Morse, 1994; Aisen, 1998). Tf, a bi-lobed protein of approximately 80 kDa, is dually responsible for essentially all iron transport within the vertebrate circulatory system (Aisen, 1998; Aisen *et* al., 2001; Mietzner and Morse, 1994) while enhancing the bactericidal activity of serum by sequestering free iron

(Bullen *et al.*, 2000). The nucleotide and deduced amino acid sequences of the Tf proteins from a number of species have been determined (see e.g. Baldwin, 1993; Retzer *et al.*, 1996) revealing that the two lobes of Tf are very similar, exhibiting greater than 60% sequence identity (Aisen *et al.*, 2001) and suggesting that the modern gene arose from a duplication event (Aisen, 1998; Aisen *et al.*, 2001). Each lobe of Tf is capable of binding one iron atom in association with a carbonate anion (Aisen, 1998; Aisen *et al.*, 1999) but circulating Tf is typically 1/3 saturated, allowing it to bind additional iron, ensuring that the circulatory system is devoid of free iron (Aisen *et al.*, 2001; Mietzner and Morse, 1994; Ratledge and Dover, 2000). The binding of iron to Tf is tight, but reversible, allowing each molecule of Tf to undergo approximately 100 to 200 cycles of iron binding, transport, and release (Aisen, 1998; Aisen *et al.*, 2001). The acquisition of Tf-bound iron by a host cell is initiated by the Tf-binding activity of Tf-receptors at the surface of the cell; these receptors are capable of discriminating between iron-loaded (holo-) Tfs or apo-Tfs and preferentially bind the former (Aisen, 1998). The Tf-receptors, with bound Tf, accumulate within clathrin-coated pits which bud from the plasma membrane as endosomes; acidification of the endosome contents (to pH ~ 5.5) then facilitates the release of the iron from the Tf (Aisen, 1998; Aisen *et al.*, 2001). Tf remains bound to its receptor during the entire process and once the iron is removed from Tf, the Tf-receptor complex is returned to the plasma membrane such that the Tf is exposed on the surface of the cell; the increased pH promotes dissociation from the receptor allowing Tf to re-enter the circulation (Aisen, 1998; Aisen *et al.*, 2001).

1.5.3 TonB-dependent receptors

In order to survive, multiply, and cause disease within a host, iron-requiring pathogens must be able to acquire iron from one or more of the iron-containing proteins of the host. There are three general types of iron-acquisition systems employed by such iron-requiring pathogens. The first system involves the synthesis and secretion of low-molecular-weight compounds, siderophores, which are capable of sequestering iron indiscriminately from host iron-containing proteins (e.g. Tf); subsequently, the ferri-siderophores are bound by specific outer membrane receptors and the iron is made available to the bacterium (Braun *et al.*, 1998). Alternatively, some pathogens are capable of acquiring their required iron (and haem, if necessary) by producing proteins that specifically bind haem or haem-containing proteins. These binding proteins can act directly as receptors, or they may be secreted (haemophores) and subsequently bound by secondary receptors, facilitating the removal and import of haem (Genco and Dixon, 2001). Finally, some pathogens are capable of producing outer membrane proteins that specifically bind, and facilitate the removal of iron from, either Tf or Lf (Gray-Owen and Schryvers, 1996). Regardless of the type of iron acquisition system employed by Gram-negative bacteria, the presence of some type of receptor within the outer membrane is required to make contact with the iron source. This requirement is absolute since the outer membrane forms a permeability barrier that prevents molecules of >600 Da from crossing it (Braun and Killmann, 1999; Koebnik, *et al.*, 2000) and most types of iron complexes, including ferrisiderophores (<1000 Da [Moeck and Coulton, 1998]) and haem (just above 600 Da [Genco and Dixon, 2001]), are larger than this. While the outer membrane receptors are capable of specifically binding their respective ligands,

they are not capable of independently effecting active transport of their ligands since there is not an energy source present in the outer membrane (Braun and Killman, 1999); the energy required for active transport must be derived from the cytoplasmic membrane.

The first clue as to how energy is transduced from the cytoplasmic to the outer membrane came from studies, in 1943 (Luria and Delbruck; cited in Postle, 1990), of *E. coli* mutants that were resistant to infection with phage T1. One of the mutants was named TonB (for T-one) and although phage T1 was able to bind to *E. coli* cells exhibiting this phenotype, the active transport of the phage to cause infection, an energy-requiring process, did not take place (Braun, 1995; Braun *et al.*, 1998; Postle, 1990). It was later revealed that such TonB mutants were incapable of importing siderophores and vitamin B_{12} and were resistant to phage ϕ80 and the B-group colicins (Braun *et al.*, 1998; Postle, 1990). TonB, encoded by *tonB*, is a 26-kDa protein capable of transferring energy from the cytoplasmic membrane to receptors residing in the outer membrane (Braun, 1995; Moeck and Coulton, 1998). For full activity, TonB must be associated with its accessory proteins, ExbB and ExbD, which are present in the cytoplasmic membrane (Braun, 1995; Moeck and Coulton, 1998). While the exact mechanism of energy transfer is unknown, many of the interactions that take place between TonB and receptors residing within the outer membrane have been elucidated. The TonB protein is anchored to the cytoplasmic membrane via the N-terminus and possesses two proline rich regions that create a rod-like structure, allowing the protein to span the periplasmic space (Braun, 1995). The C-terminus of TonB interacts with receptors at the surface of the cell (Howard *et al.*, 2001; Moeck and Letellier, 2001) and

preferentially associates with outer membrane receptors with bound ligand (Moeck and Coulton, 1998; Moeck and Letellier, 2001). Analysis of TonB-dependent outer membrane receptors revealed that they contain seven conserved regions (see e.g. Cornelissen *et al.*, 1992; Turner *et al.*, 2001) and it is generally accepted that the physical interaction takes place between TonB and the first conserved region at the N-terminus, called the TonB box (Braun, 1995; Moeck and Coulton, 1998). The TonB box takes the form of a conserved motif rather than a conserved sequence since the exact sequence varies among receptors and can be varied by mutation without affecting TonB-dependent receptor activities (Braun, 1995). A recent study of the vitamin B_{12} receptor (BtuB) revealed that substituting each residue of the TonB box individually with a cysteine residue did not result in any decrease in receptor function (Cadieux *et al.*, 2000).

While there is evidence that some bacterial species produce specific TonB homologues that are dedicated to a specific iron acquisition system (Paquelin *et al.*, 2001) or that they possess redundant *tonB* homologues (Desai *et al.*, 2000), deletion or mutation of TonB abolishes many, if not all, iron acquisition systems in *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) establishing TonB, or a functional homologue, as an essential component of many Gram-negative pathogens.

1.5.4 Siderophore-dependent iron acquisition

Of all the iron acquisition systems employed by bacteria, siderophore-dependent iron acquisition systems are most probably the best studied. Siderophores are low-molecular-weight, non-protein, iron chelators (Braun *et al.*, 1998; Griffiths, 1987; Mietzner and Morse, 1994; Ratledge and Dover, 2000) produced by most Gram-negative pathogens, with the notable exceptions of members of the families *Neisseriaceae* and *Pasteurellaceae* (Gray-Owen and Schryvers, 1996), and by non-pathogenic bacteria, including the alfalfa sybiont, *Sinorhizobium meliloti* (see e.g. Lynch *et al.*, 2001). Although there are over 500 described siderophores produced by bacteria, fungi and yeast (Ratledge and Dover, 2000), the majority of siderophores invariably belong to either the hydroxamate or catechol chemical groups (Neilands, 1995). Siderophores possess a sufficiently high affinity for iron that they are capable of removing it from Tf and Lf, but they cannot remove it from haem (Ratledge and Dover, 2000). The siderophores, with chelated iron, are subsequently bound by specific TonB-dependent receptors and transported across the outer membrane (Braun *et al.*, 1998; Griffiths, 1987).

While *E. coli* K12 has six distinct siderophore uptake systems, each recognizing a different type of siderophore, this strain is capable of synthesizing only one type of siderophore, enterobactin (also known as enterochelin) (Braun *et al.*, 1998), which belongs to the catechol family (Neilands, 1995); the remaining siderophore transport systems are responsible for the transport of ferri-siderophores produced by other organisms, including the fungal siderophore, ferrichrome (Braun *et al.*, 1998). In effect, when experiencing conditions of iron deficiency, *E. coli* cells are capable of "stealing"

siderophores produced by other species. Conversely, other bacterial species, such as *Yersinia enterocolitica* (Schubert *et al.*, 1999) and pathogenic *Neisseria* spp. (Archibald and DeVoe, 1980; Carson *et al.*, 1999), possess the system for enterobactin uptake, allowing them to "steal" the enterobactin produced by *E. coli*. Many clinical isolates of *E. coli* possess the plasmid pColV-K30, whose gene products encode systems responsible for the synthesis and uptake of an additional siderophore, aerobactin (hydroxamate-type siderophore; Neilands, 1995), which is more effective than enterobactin at removing iron from Tf under relevant physiological conditions (Bagg and Neilands, 1987). Regardless of the type of siderophore-mediated iron acquisition strategy employed, a homogenous group of bacteria must first produce the siderophore. The biosynthesis of enterobactin from its precursor chorismic acid requires the products of six genes, *entA-F* (Ratledge and Dover, 2000; Welch *et al.*, 2000), while the production of aerobactin from its precursors, lysine and citrate, requires the participation of four gene products, *iucA-D* (Bagg and Neilands, 1987; Neilands, 1992). Interestingly, it is still not known how the synthesized siderophores are exported from the cell (Braun *et al.*, 1998).

Once the siderophores have sequestered iron from the environment, the TonB-dependent outer membrane receptors are responsible for the binding and transport of the siderophore. Perhaps the best-studied siderophore receptor is FhuA, which is responsible for the binding and uptake of the fungal siderophore, ferrichrome (Braun *et al.*, 1998). The crystal structure of FhuA has recently been determined revealing that it is a 22-stranded β-barrel whose interior is blocked by an N-terminal plug (Ferguson *et al.*, 1998; Locher *et al.*, 1998). Although a deletion mutant lacked the N-terminal plug

domain but still formed the β-barrel in the outer membrane, the active transport of ferrichrome across the outer membrane remained TonB-dependent (Braun *et al.*, 1999) suggesting that regions other than the TonB box at the N-terminus may interact with TonB in "corkless" FhuA mutants (Braun *et al.*, 1999; Killmann *et al.*, 2001). FhuA mediates the transport of ferrichrome into the periplasm where it is bound by the periplasmic protein FhuD (Braun *et al.*, 1998; Braun and Killmann, 1999); the crystal structure of FhuD has also been determined (Clarke *et al.*, 2000). The siderophore is delivered to the cytoplasmic membrane protein, FhuB, which actively transports the ferrichrome using energy delivered by the ATPase protein FhuC (Braun *et al.*, 1998; Braun and Killmann, 1999). While FhuA binds and specifically transports ferrichrome, the FhuBCD proteins are essential for the binding and transportation of many other hydroxamate-type siderophores including aerobactin (Braun *et al.*, 1998). Once located in the cytoplasm, the removal of iron from most siderophores is facilitated by a ferri-reductase (Ratledge and Dover, 2000) since most siderophores possess an affinity for Fe^{2+} that is much lower than that for Fe^{3+} (Neilands, 1995). The reductive release of iron from enterochelin, however, first requires the hydrolysis of the molecule by a specific esterase (Griffiths, 1987; Ratledge and Dover, 2000).

1.5.4.1 Regulation of siderophore-dependent iron acquisition systems

Regulation of the aerobactin operon on pColV-K30 is mediated exclusively by the Fur protein that binds to the promoter and represses the transcription of the operon when iron is present at sufficiently high amounts (de Lorenzo *et al.*, 1987; Escolar *et al.*, 2000). This type of regulation is logical since the organism produces both the

siderophore to sequester the available iron and the system for siderophore uptake. However, *E. coli* and other organisms can produce receptors for heterologous siderophores. Since the organisms only produce the uptake system, but not the siderophore itself, regulation by Fur alone would allow the expression of uptake systems even when the siderophore is not present. To circumvent such a problem, regulation must be more complex. For example, *E. coli* possesses the uptake system allowing it to use exogenously provided ferric citrate as an iron source, but the system is only fully expressed when iron is limiting and ferric citrate is present (Braun, 1997). The binding of ferric citrate to the ferric citrate receptor (FecA) in the outer membrane induces transcription of the *fecABCDE* transport genes (see e.g. Braun, 1997; Angerer and Braun, 1998; Enz *et al.*, 2000; Welz and Braun, 1998). Signal transduction involves FecA, FecI, which is cytoplasmic, and FecR which is located in the cytoplasmic membrane; FecR is believed to be involved in the transfer of the signal from FecA to FecI and in the activation of FecI (Enz *et al.*, 2000; Welz and Braun, 1998). The regulatory genes, *fecIR*, are located upstream of the *fec* transport genes and while *fecIR* and *fecABCDE* are transcribed separately, both are regulated by Fur (Angerer and Braun, 1998). FecI is a sigma factor belonging to the ECF (extracytoplasmic functions) subfamily of σ^{70} factors and promotes transcription from the promoter upstream of *fecA* (Angerer *et al.*, 1995; Braun *et al.*, 1998). Similar systems appear to be involved in the positive regulation by siderophores of receptor expression in pseudomonads (see e.g., Braun, 1997; Crosa, 1997; Braun *et al.*, 1998) but note that the enterobactin-inducible production of the enterobactin receptor of *P. aeruginosa* is mediated by a Fur-regulated two-component system (Dean and Poole, 1993; Dean *et al.*, 1996).

1.5.5 Contact-dependent iron acquisition

1.5.5.1 Haem- and haemoprotein-binding proteins

Approximately 60% of the iron within a host is associated with haemoglobin (Mietzner and Morse, 1994). While haem can serve as a source of iron, many pathogens cannot synthesize haem and require haem *per se*. *H. influenzae*, for instance, lacks essentially the entire haem biosynthetic pathway save the last enzyme, ferrochelatase, which catalyzes the insertion of iron into protoporphyrin IX resulting in the formation of haem (Loeb, 1995). Although it would appear that some pathogens can acquire free haem *in vitro* by a TonB- and receptor-independent mechanism (Chen *et* al., 2002; Thomas *et al.*, 1998), free haem is rapidly complexed to protein carriers *in vivo* (e.g. haemopexin [Mietzner and Morse, 1994]) effectively forcing many pathogens to evolve an array of different systems capable of acquiring haem (Genco and Dixon, 2001).

Two general mechanisms for acquiring haem have been identified by biochemical and/or genetic methods in 19 different bacterial species (as reviewed in Genco and Dixon, 2001; May *et al.*, 2000). The first involves the production of TonB-dependent, outer membrane receptors that can bind haemoproteins directly, while the second involves the production of extracellular soluble proteins, called haemophores, that sequester haem and deliver it to TonB-dependent receptors at the outer membrane surface (Genco and Dixon, 2001; Wandersman and Stojiljkovic, 2000). The TonB-dependent haemoglobin and/or haemoglobin-haptoglobin receptors of *Neisseria* and *Haemophilus* spp., with molecular masses ranging from 85 to 120 kDa, are perhaps some of the best studied (see e.g. Chen *et al.*, 2002; Elkins, 1995; Jin *et al.*, 1999; Lewis and Dyer, 1995; Maciver *et al.*, 1996; Ren *et al.*, 1998; Stojiljkovic *et al.*, 1996).

Haemoglobin binding by these receptors can be non-specific, suggesting the involvement of the haem moiety in binding (Elkins, 1995), while binding by others demonstrates some degree of specificity towards the haemoglobin of the host suggesting that a motif within the globin moiety is recognized and bound by the receptor (Stojiljkovic *et al.*, 1996). In addition to the single component receptors, *Neisseria* spp. produce a bipartite receptor composed of an 85-kDa TonB-dependent outer membrane protein, HpuB, and a 35-kDa lipoprotein, HpuA, analogous to the Tf (TbpA and TbpB) and Lf (LbpA and LbpB) receptors [see below (Genco and Dixon, 2001; Lewis *et al.*, 1997; Schryvers and Stojiljkovic, 1999)]; both components are required for the utilization of haemoglobin and haemoglobin-haptoglobin as iron sources (Chen *et al.*, 2002; Lewis and Dyer, 1995; Lewis *et al.*, 1998b; Schryvers and Stojiljkovic, 1999). The 100-kDa protein, HxuA, produced by *H. influenzae* is responsible for sequestering haem from haem-haemopexin complexes (Cope *et al.*, 1998), an attribute possessed only by *H. influenzae* (Genco and Dixon, 2001). HxuA may be located at the surface of the cell, although it does not function as a TonB-dependent receptor, or it may be released into the surroundings (Cope *et al.*, 1998) in much the same way as the haemophores that have been identified in other Gram-negative pathogens including *Serratia marcescens* (Létoffé *et al.*, 1994, 1999), *P. aeruginosa* (Létoffé *et al.*, 1998) and *Y. pestis* (Rossi *et al.*, 2001). In fact, exogenously supplied HxuA allows an *hxuA* mutant to acquire haem from haem-haemopexin (Cope *et al.*, 1998) strengthening the argument that HxuA functions as a haemophore. The haemophore system is comprised of a haemophore that is secreted into the extracellular milieu by an ABC exporter, where it is capable of sequestering either free or

haemoglobin-bound haem (Wandersman and Stojiljkovic, 2000), and an outer membrane TonB-dependent receptor that binds the haemophore and transports the intact haem molecule into the cell (Létoffé *et al.*, 1999; Wandersman and Stojiljkovic, 2000). This strategy for acquiring haem is reminiscent of the siderophore-dependent mechanism for iron acquisition but it would appear that the haemophore receptors are unable to obtain haem from the haemophores secreted by other species since exogenously supplied haemophore from *Y. pestis* cannot complement a haemophore production mutant of *S. marcescens* (Rossi *et al.*, 2001); also, all organisms studied to date appear to possess the genes encoding both the receptor and the haemophore (see e.g. Létoffé *et al.*, 1998, 1999; Rossi *et al.*, 2001). Regardless of the type of system used, the intact haem molecule is transported intact into the cytoplasm of the bacterium, much like ferri-siderophores (Lewis *et al.*, 1998b; Wandersman and Stojiljkovic, 2000). Once inside the cell, iron is removed from haem and although the fate of intracellular haem is not well understood, the destruction of the haem molecule by a haem oxygenase appears to be an essential step in acquiring haem-bound iron in the pathogenic *Neisseriaceae* (Zhu *et al.*, 2000) and the Gram-positive pathogen, *Corynebacterium diphtheriae* (Schmitt, 1997); *H. influenzae*, on the other hand, may possess reverse ferrochelatase activity allowing the removal of iron from haem without destroying the haem molecule (Loeb, 1995).

1.5.5.2 Regulation of expression of haem- and haemoprotein-binding proteins

Regulation of the haemophore systems appears to be mediated solely by the levels of iron since the haemophores of *S. marcescens* (Létoffé *et al.*, 1994), *P. aeruginosa* (Létoffé *et al.*, 1998) and *Y. pestis* (Rossi *et al.*, 2001) are produced under conditions of iron restriction and the haemophore operons of all three organisms contain putative Fur boxes in the promoter region (Létoffé *et al.*, 1998, 1999; Rossi *et al.*, 2001). The situation with *Neisseria* spp. is somewhat more complex; the expression of both the HmbR haemoglobin receptor and the bipartite HpuAB system responsible for acquiring haem from haemoglobin or haemoglobin-haptoglobin are repressible by iron (Lewis *et al.*, 1999; Stojiljkovic *et al.*, 1996) but the expression of both may be affected by phase variation involving a poly G tract located within the coding region of *hmbR* or *hpuA* of the *hpuAB* operon (Chen *et al.*, 1998; Lewis *et al.*, 1999; Richardson and Stojiljkovic, 1999). The addition of a G to this tract can alter the reading frame introducing a premature stop codon, resulting in a truncated and non-functional protein (Chen *et al.*, 1998; Lewis *et al.*, 1999; Richardson and Stojiljkovic, 1999). The regulatory mechanisms within *Haemophilus* spp. are perhaps the most complex as these organisms have an absolute haem requirement in addition to being able to acquire iron from haem. While it would appear that the expression of various haemoprotein-binding proteins in *Haemophilus ducreyi* (Thomas *et al.*, 1998) and *H. influenzae* (Whitby *et al.*, 1997) are repressible by haem, elemental iron alone may (Hasan *et al.*, 1997) or may not (Maciver *et al.*, 1996) repress expression of haemoprotein receptors in *H. influenzae*. In addition to being haem- and possibly, iron-repressible, the expression of

other haemoprotein receptors, such as the haemoglobin/ haemoglobin-haptoglobin binding protein, HgpA, in *H. influenzae* may also be regulated by a form of phase variation where the removal of one or two CCAA repeats within *hgpA* introduces a premature stop codon within the coding sequence (Jin *et al.*, 1999). Regardless of the type of regulation, it is apparent that organisms such as *H. influenzae*, which have an absolute requirement for haem (or protoporphyrin IX [Loeb, 1995]), must be able to express haem acquisition systems *in vivo* and the transcription of such genes, although not translation, during acute otitis media has been demonstrated for two genes, *hxuA* and *hgpA* (Whitby *et al.*, 1997).

1.5.5.3 Tf- and Lf-binding proteins

Archibald and DeVoe (1979) demonstrated that acquisition of iron from human Tf by *N. meningitidis* is contact-dependent; free Tf in the growth medium could serve as an iron source whereas Tf contained in a dialysis bag could not. Shortly thereafter, it was demonstrated that *Neisseria gonorrhoeae* can also obtain iron specifically from human Tf (Mickelson and Sparling, 1981) and that both *N. meningitidis* and *N. gonorrhoeae* are capable of acquiring Lf-bound iron (Mickelson *et al.*, 1982). As is the case for *N. meningitidis* (Archibald and DeVoe, 1980), *N. gonorrhoeae* does not produce siderophores (West and Sparling, 1985) and acquires Tf- and Lf-bound iron in a contact-dependent manner (McKenna *et al.*, 1988). The human pathogen, *Moraxella catarrhalis* (Campagnari *et al.*, 1994), and the bovine pathogen, *Moraxella bovis* (Bonnah *et al.*, 1995), are both able to use specifically host Tf and Lf as iron sources by siderophore-independent mechanisms, establishing members of the *Neisseriaceae*

family as the only ones possessing receptor-mediated mechanisms for acquiring both Tf- and Lf-bound iron (Gray-Owen and Schryvers, 1996). Furthermore, *M. bovis* is the only described veterinary pathogen possessing the ability to acquire iron from host Lf by a receptor-mediated mechanism (Bonnah *et al.*, 1995). *H. influenzae* is able to acquire iron from host Tf but not from Lf (Harrington and Sparling, 1985; Pidcock *et al.*, 1988) and where studied, this is also the case for all other pathogenic members of the *Pasteurellaceae* family including *Actinobacillus pleuropneumoniae* (D'Silva *et al.*, 1995; Gonzalez *et al.*, 1990; Niven *et al.*, 1989), *Haemophilus parasuis* (Charland *et al.*, 1995), *Mannheimia haemolytica* (Ogunnariwo and Schryvers, 1990; Yu *et al.*, 1992), *P. multocida* (Ogunnariwo *et al.*, 1991; Veken *et al.*, 1996) and *H. somnus* (Ogunnariwo *et al.*, 1990; Yu *et al.*, 1992). While there are examples of bacteria not belonging to the *Neisseriaceae* or *Pasteurellaceae* families that are capable of using Lf or Tf as an iron source, presumably by a receptor-mediated mechanism (Dhaenens *et al.*, 1997; Husson *et al.*, 1993; Jarosik *et al.*, 1998; Jarosik and Land, 2000; Modun *et al.*, 1998), such mechanisms either have not been as extensively characterized or have been found to be unrelated (Modun and Williams, 1999) to the systems employed by members of the *Neisseriaceae* or *Pasteurellaceae* families; consequently, these systems will not be discussed further.

The receptor-mediated acquisition of Tf- or Lf-bound iron is unique in that the pathogens that can accomplish this are capable of acquiring iron only from host Tf or Lf (see e.g., Bonnah *et al.*, 1995; Charland *et al.*, 1995; Niven *et al.*, 1989; Ogunnariwo *et al.*, 1990; Schryvers and Gonzalez, 1990; Schryvers and Morris, 1988b; Yu *et al.*, 1992). As examples, *A. pleuropneumoniae*, a porcine pathogen, is capable of acquiring

iron specifically from porcine, but not from human or bovine Tf (Niven *et al.*, 1989) and *N. meningitidis*, strictly a human pathogen, is capable of acquiring iron from human but not from bovine Lf (Schryvers and Morris, 1988b). In most cases, such pathogens are therefore able to acquire iron only from a single species of Tf (or Lf). *M. haemolytica*, however, is capable of binding and acquiring iron from bovine, ovine, and goat Tfs (Yu *et al.*, 1992). Solid phase binding assays have also demonstrated that *H. agni* (Yu and Schryvers, 1994) and *P. multocida* (Ogunnariwo and Schryvers, 2001) are capable of binding specifically bovine, ovine and goat Tfs implying that all three can be used as an iron source, but note that growth with ovine and goat Tfs has not been demonstrated. Such solid phase binding assays, employing horseradish peroxidase (HRP)-conjugated Tf or Lf, were used to demonstrate the iron-repressible nature of Tf- and Lf-binding activity for a variety of pathogens and, where investigated, the spectrum of Tfs or Lfs that are bound are identical to those that can be used as an iron source (Schryvers and Lee, 1988; Morton and Williams, 1990; Ogunnariwo *et al.*, 1990; Ogunnariwo and Schryvers, 1990; Yu *et al.*, 1992). In competition experiments, where excess native Lfs or Tfs compete with the HRP-conjugated Lf or Tf that is normally bound by the pathogen, it was demonstrated that the receptor(s) can be saturated and additionally, that Lfs or Tfs not normally bound by the pathogen cannot effectively compete for receptor binding sites (Morton and Williams, 1990; Ogunnariwo *et al.*, 1990; Schryvers, 1988; Schryvers and Morris, 1988a,b). Furthermore, it was demonstrated that the Lf and Tf receptors of pathogenic *Neisseria* spp. are distinct entities, since excess native human Tf could not compete with HRP-conjugated human Lf and vice versa (Lee and Schryvers, 1988; Schryvers and Morris, 1988a,b).

The receptors responsible for the binding of Tf are localized to the outer membrane (Ala'Aldeen *et al.*, 1993, Morton and Williams, 1990) and affinity isolation experiments allowed the identification of two proteins, initially named transferrin binding proteins 1 (Tbp1) and 2 (Tbp2) and subsequently, as TbpA and TbpB, which were responsible for binding Tf (Gray-Owen and Schryvers, 1996). The TbpA proteins are integral, outer membrane, TonB-dependent proteins, while the TbpB proteins are surface-exposed lipoproteins that are presumably anchored to the outer membrane (see e.g., Cornelissen and Sparling, 1994; Gray-Owen and Schryvers, 1996). The TbpA proteins typically range from 93 to 105 kDa in size while the sizes of the TbpB proteins are somewhat more variable, ranging from 56 to 86 kDa (Charland *et al.*, 1995; D'Silva *et al.*, 1995; Gonzalez *et al.*, 1990; Ogunnariwo *et al.*, 1990; Ogunnariwo and Schryvers, 1990; Schryvers, 1989; Schryvers and Lee, 1988). Aside from their differences in size, the TbpB proteins from many species can be distinguished from the TbpAs by their abilities to bind ^{125}I-labeled or HRP-conjugated Tf following SDS-PAGE and electrophoretic transfer to nitrocellulose or polyvinylidene difluoride (PVDF) (Gonzalez *et al.*, 1990; Griffiths *et al.*, 1990; Myers *et al.*, 1998; Schryvers, 1989; Schryvers and Lee, 1988; Schryvers and Morris, 1988a; Stevenson *et al.*, 1992). The TbpA-TbpB bipartite receptor system is used by essentially all pathogens that acquire Tf-bound iron in a contact-dependent manner (see e.g., Cornelissen and Sparling, 1994; Gray-Owen and Schryvers, 1996) with the notable exception of *P. multocida*; *P. multocida* produces a TbpA homologue (albeit a smaller one with a molecular mass of 82 kDa), but so far, genetic and biochemical approaches have failed to establish the existence of a TbpB homologue (Ogunnariwo *et al.*, 1991; Ogunnariwo

and Schryvers, 2001). Initially, a single high-molecular-weight lactoferrin-binding protein (101 to 105 kDa) was isolated from the pathogenic *Neisseria* spp. (Lee and Bryan, 1989; Schryvers and Lee, 1988; Schryvers and Morris, 1988b) and by convention, was named LbpA (Gray-Owen and Schryvers, 1996). A second lactoferrin-binding protein was inferred from genetic evidence; the predicted product of a gene upstream of *lbpA* exhibited homology to TbpB (Pettersson *et al.*, 1994a) and putative LbpB proteins of approximately 85 kDa were subsequently isolated from pathogenic members of the *Neisseriaceae* family (Bonnah *et al.*, 1995). While the ~85 kDa protein isolated from *N. meningitidis* is probably the LbpB homologue (Bonnah *et al.*, 1998), the 85 kDa protein isolated from *M. catarrhalis* was later identified as CopB and a 95 kDa protein that co-migrated with LbpA on SDS-PAGE was identified as the putative LbpB protein (Bonnah *et al.*, 1998). This result was subsequently confirmed when the structural genes for LbpA and LbpB from *M. catarrhalis* were cloned and sequenced (Du *et al.*, 1998). Interestingly, while *M. catarrhalis copB* mutants are capable of binding HRP-Tf and -Lf (Aebi *et al.*, 1996), their ability to acquire iron from these proteins is severely diminished (Aebi *et al.*, 1996; Bonnah *et al.*, 1998). In addition to demonstrating some degree of homology with TbpB proteins, the LbpB proteins also possess the ability to bind HRP-conjugated Lf following SDS-PAGE and electrophoretic transfer (Du *et al.*, 1998; Pettersson *et al.*, 1998).

1.5.5.4 Genetic organization and regulation of *tbp* and *lbp* genes

The TbpA, TbpB, LbpA and LbpB proteins are encoded by their respective genes, *tbpA*, *tbpB*, *lbpA* and *lbpB* (Gray-Owen and Schryvers, 1996). In essentially

every case, the *tbpB* or *lbpB* gene precedes the *tbpA* or *lbpA* gene (Anderson *et al.*, 1994; Biswas *et al.*, 1999; Bonnah and Schryvers, 1998; Du *et al.*, 1998 Gonzalez *et al.*, 1995; Gray-Owen *et al.*, 1995; Legrain *et al.*, 1993; Lewis *et al.*, 1998a; Ogunnariwo *et al.*, 1997; Pettersson *et al.*, 1998). There are only two species where this arrangement does not apply. In *P. multocida*, which does not appear to possess a TbpB or *tbpB* homologue, the *tbpA* gene is flanked by genes encoding a leucyl-tRNA synthetase (upstream) and an IS-like element (downstream) (Ogunnariwo and Schryvers, 2001) and in *M. catarrhalis*, the *tbpA* gene is upstream of *tbpB* and the two genes are separated by an unknown *orf* (Myers *et al.*, 1998). Although putative promoters and (or) Fur boxes have been identified immediately upstream of *lbpA* in pathogenic *Neisseria* spp. (Biswas and Sparling, 1995; Lewis *et al.*, 1998a; Pettersson *et al.*, 1994a), the *lbpB* and *lbpA* coding regions overlap (Biswas *et al.*, 1999; Lewis *et al.*, 1998a) and the *lbpBA* genes are transcribed as a polycistronic message from the promoter upstream of *lbpB* (Biswas *et al.*, 1999; Bonnah and Schryvers, 1998; Lewis *et al.*, 1998a). The *lbpB* and *lbpA* genes in *M. catarrhalis* are separated by 184 bp and putative promoters and Fur boxes have been identified upstream of both genes; however, whether the two genes are transcribed independently, or as an operon, has not been experimentally determined (Du *et al.*, 1998). Although the *tbpB* and *tbpA* genes do not overlap, putative promoters and Fur boxes were identified only upstream of *tbpB* (Anderson *et al.*, 1994; Gonzalez *et al.*, 1995; Gray-Owen *et al.*, 1995; Legrain *et al.*, 1993; Ogunnariwo *et al.*, 1997) suggesting that the two genes are transcribed as an operon. However, experimental evidence demonstrating that *tbpBA* are transcribed as an operon has only been obtained with *N. gonorrhoeae* (Anderson *et al.*, 1994; Ronpirin *et al.*, 2001). Interestingly,

although the *tbpB* and *tbpA* genes are transcribed as an operon, when *N. gonorrhoeae* is grown under iron-restricted conditions, the ratio of *tbpB* to *tbpA* specific mRNA is 2:1; the mechanism resulting in an increased amount of *tbpB* transcript, however, has not been elucidated (Ronpirin *et al.*, 2001).

The identification of putative Fur boxes within the promoter regions of a number of *lbp* and *tbp* genes (Anderson *et al.*, 1994; Du *et al.*, 1998; Gonzalez *et al.*, 1995; Gray-Owen *et al.*, 1995; Ogunnariwo *et al.*, 1997; Pettersson *et al.*, 1994a, 1998), the iron-repressible nature of the Tbp and Lbp proteins (see above), and the identification of *fur* homologues in many members of the *Neisseriaceae* and *Pasteurellaceae* (see e.g., Thomas and Sparling, 1994; May *et al.*, 2001), suggest that the expression of such proteins is Fur-regulated at the transcriptional level. However, direct evidence demonstrating iron-regulated transcription of such genes has only been demonstrated for the *lbp* genes of pathogenic *Neisseria* spp. (Bowler *et al.*, 1999; Lewis *et al.*, 1998a) and the *tbp* genes of *N. gonorrhoeae* (Ronpirin *et al.*, 2001). It would appear that the iron-repressible nature of the *tbp* genes in *N. gonorrhoeae* is due to the Fur protein since a *fur* missense mutant generated by manganese selection and grown under iron-replete conditions, expressed elevated levels of TbpA and TbpB when compared to the wild-type strain (Thomas and Sparling, 1996).

1.5.5.5 Mechanism of Tf and Lf binding and removal of iron

Tf receptors are localized to the outer membrane of Gram-negative bacteria (Ala'Aldeen *et al.*, 1993). The iron is apparently removed from Tf at the surface of the cell since growth of *N. gonorrhoeae* with double-labeled ^{59}Fe-^{125}I-human Tf resulted in

uptake of ^{59}Fe without incorporation of ^{125}I (McKenna *et al.*, 1988). This is in contrast to eukaryotic cells which must first internalize Tf before removing the iron (Aisen, 1998). The binding of Tf to the bacterial Tf receptors does not require the involvement of the N-linked oligosaccharides present on Tf (Padda and Schryvers, 1990) and while the binding of Tf is an energy (or TonB)-independent process, the release of Tf from the receptor requires energy (Cornelissen *et al.*, 1997). The TbpA proteins are TonB-dependent receptors based on homology (see e.g., Cornelissen *et al.*, 1992) and functional studies with *tonB* mutants (see e.g., Biswas *et al.*, 1997; Stojiljkovic and Srinivasan, 1997) and based on the recently published crystal structures of the *E. coli* siderophore receptors, FhuA (Ferguson *et al.*, 1998; Locher *et al.*, 1998) and FepA (Buchanan *et al*, 1999), the proposed structure of TbpA (Boulton *et al.*, 2000; Masri and Cornelissen, 2002; Ogunnariwo and Schryvers, 2001) and LbpA (Prinz *et al.*, 1999) is a 22 strand β-barrel whose interior is blocked by an N-terminal plug. Of the 11 surface-exposed loops created by the 22 strands, loops 4 and 5 are involved in Tf-binding and are needed to acquire Tf-bound iron (Boulton *et al.*, 2000; Masri and Cornelissen, 2002). Furthermore, native, non-denatured LbpA (Prinz *et al.*, 1999) and TbpA (Boulton *et al.*, 1998) form complexes of ~200 kDa suggesting the formation of a dimer complex. Studies with isogenic *tbpB* and *tbpA* mutants demonstrated that both TbpA and TbpB are capable of independently binding Tf (Anderson *et al.*, 1994; Gray-Owen *et al.*, 1995; Irwin *et al.*, 1993; Luke and Campagnari, 1999); however, only the TbpA protein is absolutely essential for the uptake of iron from Tf and it would appear that the TbpB protein plays an accessory role improving the efficiency and/or ability of cells to acquire Tf-bound iron (Anderson *et al.*, 1994; Cornelissen *et al.*, 1992; Gray-Owen *et*

al., 1995; Luke and Campagnari, 1999). Essentially identical results were obtained with *lbpB* and *lbpA* isogenic mutants; while both proteins are capable of binding Lf, only LbpA is necessary for the acquisition of Lf-bound iron (Biswas *et al.*, 1999; Bonnah and Schryvers, 1998; Bonnah *et al.*, 1999 Lewis *et al.*, 1998a; Pettersson *et al.*, 1998). Interestingly, the introduction of *tbpA* (Cornelissen *et al.*, 1993) or *lbpA* (Pettersson *et al.*, 1994b) into *E. coli* allowed this organism to specifically bind Tf or Lf, respectively, and the replacement of the *tbpBA* genes of *N. meningitidis* with those of *A. pleuropneumoniae*, allowed the former organism to specifically bind and acquire iron from porcine Tf (Litt *et al.*, 2000).

Although early studies indicated that apo- and 100% iron-saturated human Tf are able to compete equally for Tf-binding sites on the surface of *N. meningitidis* (Tsai *et al.*, 1988), recent data suggest that the TbpB protein is capable of discriminating between apo- and holo-Tf (Boulton *et al.*, 1998; Cornelissen and Sparling, 1996). Studies with isogenic *tbpA* and *tbpB* mutants of *N. meningitidis* revealed that both TbpA and TbpB bind predominantly to a region located in the proteolytically derived C-terminal lobe of human transferrin (Alcantara *et al.*, 1993) and using human/bovine Tf constructs, the binding region within the C-terminal lobe was found to reside between amino acids 364 and 588 (Retzer, *et al.*, 1996). While the Tf-receptors of most other pathogens also bind the C-lobe of Tf (Alcantara *et al.*, 1993; Yu and Schryvers, 1994), the *P. multocida* Tf receptor, consisting of only a TbpA protein, binds to the N-lobe of Tf (Ogunnariwo and Schryvers, 2001). Further studies with recombinant N- or C-terminal TbpB fusion proteins suggest that while the N-terminal half of TbpB preferentially interacts with the C-lobe of Tf, both lobes of TbpB are capable of binding

regions located in the N- or C-lobe of Tf (Retzer *et al.*, 1999). The bacterial Lf-receptors appear to bind equally well to both the N- and C-lobes of Lf (Yu and Schryvers, 1993); however, the individual contributions of the LbpA and LbpB proteins to this phenomenon remain obscure since this study (Yu and Schryvers, 1993) preceded the identification of LbpB (Gray-Owen and Schryvers, 1996).

There is a physical interaction between the TbpA and TbpB proteins (Fuller *et al.*, 1998) and the two proteins form a complex of ~300 kDa suggesting that one molecule of TbpB associates with dimerized TbpA (Boulton *et al.*, 1998). The TbpB protein preferentially binds holo-Tf and presents it to the TbpA proteins which can then effect the active transport of iron into the periplasm of the bacterial cell (Boulton *et al.*, 1999). However, for the iron to be of any use to the bacterium, it must be transported into the cytoplasm. Iron removed from Tf or Lf is delivered to the periplasm and transported across the cytoplasmic membrane by the products of the *fbpABC* gene clusters that have been found in pathogenic *Neisseria* spp. (Adhikari *et al.*, 1996; Khun *et al.*, 1998), *M. haemolytica* (Kirby *et al.*, 1998) and *H. influenzae* (Adhikari *et al.*, 1995); although the genes are arranged as putative operons, only the *fbpABC* genes of *N. meningitidis* have been demonstrated to be transcribed as a single transcriptional unit (Khun *et al.*, 2000). The *fbpA* genes encode proteins capable of binding a single Fe^{3+} ion with an affinity comparable to the N-lobe of Tf (Adhikari *et al.*, 1995; Taboy *et al.*, 2001). FbpA delivers iron to a cytoplasmic permease, encoded by *fbpB*, which translocates the iron into the cytoplasm using energy supplied by the nucleotide-binding protein encoded by *fbpC* (see e.g., Adhikari *et al.*, 1996); although a *N. gonorrhoeae* *fbpC* mutant is capable of obtaining iron from Tf (Sebastian and Genco, 1999), an

alternative nucleotide binding protein within the cell may functionally substitute for FbpC, complementing the mutation. Consistent with their role in iron acquisition, the *fbp* genes are transcribed under conditions of iron restriction (Forng *et al.*, 1997; Khun *et al.*, 2000) and in *N. gonorrhoeae*, Fur binds to the promoter region of *fbpA* (Desai *et al.*, 1996) and increased amounts of FbpA are produced by a *fur* missense mutant (Thomas and Sparling, 1996).

1.5.5.6 Tf- and Lf-binding proteins as vaccine components

While the majority of the studies pertaining to the acquisition of iron by pathogenic members of the *Neisseriaceae* and *Pasteurellaceae* have been performed *in vitro*, the contribution of such iron acquisition systems to the virulence of the pathogen is clear. The mortality among mice experimentally infected with *N. meningitidis* is significantly increased if human Tf or Lf is included in the inoculum (Holbein, 1981; Schryvers and Gonzalez, 1989) and *N. gonorrhoeae tbp* mutants are unable to cause disease in human males (Cornelissen *et al.*, 1998). The Tbps of *H. influenzae* are known to be expressed *in vivo* since the transcription of *tbpA* has been verified by performing reverse transcription (RT)-PCR on samples taken from individuals with acute otitis media (Whitby *et al.*, 1997) and convalescent-phase human sera from patients diagnosed with *H. influenzae* type b meningitis react with the Tbps (Holland *et al.*, 1992). Similarly, immune sera from patients recognized LbpB, but not LbpA (Yu *et al.*, 1999). As the Tf-receptors are localized to the surface of the cell (Ala'Aldeen *et al.*, 1993), the utility of the Tbps (and Lbps) as vaccine components has been investigated. It would appear that TbpB is more likely to elicit the production of protective antibodies

than is TbpA (Ala'Aldeen *et al.*, 1994; Potter *et al.*, 1999; Yu *et al.*, 1999). While recombinant TbpB of *M. catarrhalis* elicits bactericidal antibody activity (Myers *et al.*, 1998), sera from a patient infected with one strain of *M. catarrhalis* failed to recognize the TbpBs of another, demonstrating the antigenic heterogeneity among TbpBs from different strains of this species (Yu *et al.*, 1999) and effectively limiting the use of a single species of TbpB as a vaccine component. On the other hand, sera from cattle exposed to a single species of TbpB from *M. haemolytica* (Potter *et al.*, 1999) and sera from an individual infected with *N. meningitidis* (Ala'Aldeen *et al.*, 1994) demonstrated cross reactivity against the TbpBs of other serotypes or species (Ala'Aldeen *et al.*, 1994; Potter *et al.*, 1999). With these studies in mind, the feasibility of using newly discovered Tbps or Lbps as vaccine components is undoubtedly worthy of investigation.

1.6 Aims of the research project

H. ovis is closely related to *H. agni* and *H. somnus*. At the outset of this project it was known that *H. agni* can bind, specifically, ovine, bovine and goat Tfs (Yu and Schryvers, 1994) and that *H. somnus* can bind, and acquire iron from, only bovine Tf (Yu *et al.*, 1992) but comparable information was not available for organisms referred to as *H. ovis* and hence, with respect to iron acquisition, the extent to which *H. ovis* resembles *H. somnus* or *H. agni* remained unknown. The purpose of this research project was therefore to determine if *H. ovis* is capable of acquiring Tf-bound iron and if so, to characterize such ability.

Chapter 2. Production of transferrin receptors by *Histophilus ovis*: three of five strains require two signals

This chapter was adapted from the following:

Ekins, A., and Niven, D.F. 2001. Production of transferrin receptors by *Histophilus ovis*: three of five strains require two signals. Can. J. Microbiol. **47:** 417-423.

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2.1 Summary

Five strains of *Histophilus ovis* (9L, 642A, 714, 5688T and 3384Y) were investigated with respect to iron acquisition. All strains used ovine, bovine and goat, but not porcine or human, Tfs as iron sources for growth. In solid phase binding assays, total membranes from only two (9L and 642A) of the five strains, grown under iron-restricted conditions, were able to bind Tfs (ovine, bovine and goat, but not porcine or human). However, when the organisms were grown under iron-restricted conditions in the presence of bovine Tf, total membranes from all strains exhibited Tf binding (as above); competition experiments demonstrated that all three Tfs (ovine, bovine and goat) were bound by the same receptor(s). Membranes from organisms grown under iron-replete conditions in the presence or absence of bovine Tf failed to bind any of the test Tfs. An affinity isolation procedure allowed the isolation of two putative Tf-binding polypeptides (78 and 66 kDa) from total membranes of strains 9L and 642A grown

under iron-restricted conditions, and from membranes of all strains if the growth medium also contained Tf. It is concluded that all strains tested acquire Tf-bound iron by means of siderophore-independent mechanisms involving surface receptors analogous to the Tf-binding proteins (TbpA and TbpB) found in comparable organisms; while iron restriction alone is sufficient to promote the expression of these proteins by strains 9L and 642A, their production by strains 714, 5688T and 3384Y appears to require two signals, iron restriction and the presence of Tf.

2.2 Introduction

Although iron is plentiful in animal hosts, the extracellular environments encountered by pathogens *in vivo* are ones of iron restriction due to the presence of host iron-binding proteins such as Tf and Lf (Griffiths 1987). To circumvent such conditions, pathogenic members of the *Pasteurellaceae* and *Neisseriaceae* acquire iron from host Tfs by means of Tf-receptors, comprised, in most cases, of the proteins known as TbpA and TbpB (see e.g., Williams and Griffiths, 1992; Gray-Owen and Schryvers, 1996; Byers and Arceneaux, 1998). While the Tf-receptors of most organisms bind a single species of Tf that reflects their host specificity (see e.g. Yu *et al.* 1992), the receptor protein(s) of *M. haemolytica*, *P. multocida* and *H. agni* are capable of binding ovine, bovine and goat Tfs (Ogunnariwo and Schryvers, 2001;Yu *et al.*, 1992; Yu and Schryvers, 1994). *H. agni*, *H. somnus* and *H. ovis* are closely related but in contrast to *H. agni*, *H. somnus* can bind, and acquire iron from, only bovine Tf (Yu *et al.*, 1992). Unfortunately, comparable information is not available for the organisms referred to as *H. ovis* and hence, with respect to iron acquisition, the extent to

which *H. ovis* resembles *H. somnus* or *H. agni* remains unknown. The purpose of the present study was therefore to investigate iron acquisition by ovine isolates of organisms that appeared to be representative of *H. ovis*. The selected isolates appear to be very similar based on DNA hybridization data (Walker *et al.*, 1985) and for the sake of clarity, all will be referred to as *H. ovis*.

2.3 Materials and methods

2.3.1 Organisms

The *H. ovis* strains used in this study were provided by Dr. R.L. Walker (School of Veterinary Medicine, University of California at Davis) and were described by Walker *et al.* (1985). Storage cultures and inocula were stored frozen (−80°C) as small volumes (~1.5 mL) of late exponential phase cultures supplemented with glycerol (50 mL of a 75% (w/v) solution per 200-mL culture); the growth medium was HEPES-buffered tryptone-yeast extract (TYE-H; Niven *et al.*, 1989) supplemented, just prior to inoculation, with thiamine monophosphate (to 10 μg/mL), L-cysteine (to 0.5 mg/mL) and $NaHCO_3$ (to 10 mM) and is referred to as supplemented TYE-H (sTYE-H).

2.3.2 Transferrins and conjugates

Bovine and human apo-Tfs were from Calbiochem and porcine Tf was from First Link (West Midlands, UK). Ovine and goat Tfs were purified from their respective sera (Gibco BRL) as described by Niven *et al.* (1989) for porcine Tf except that the proteins were eluted from the DEAE-Sepharose CL-6B using 75 mM NaCl, 25 mM Tris-HCl, pH 8.6 and 150 mM NaCl, 25 mM Tris-HCl, pH 7.6 (Monet-Kuntz *et al.*, 1992). The Tfs were saturated with iron (to 60-90% saturation) and then dialyzed as

described by Caldwell and Archibald (1987). Following dialysis, the preparations were sterilized by filtration (0.2 μm pore size) and the iron-saturation levels checked using the method of Mazurier and Spik (1980).

HRP-conjugated human Tf was from Pierce Chemical. The conjugation of HRP to ovine, bovine, goat and porcine Tfs was accomplished using a peroxidase labelling kit (Boehringer Mannheim) and the instructions provided by the manufacturer and biotinylation of Tfs was accomplished using the method of Schryvers and Morris (1988b) as modified by Ricard *et al.* (1991).

2.3.3 Plate assays

Organisms (0.1-mL volumes) were spread on solid medium containing sTYE-H, 50 μM ethylenediamine di-*o*-hydroxyphenylacetic acid (EDDA; Sigma) and 1.6% (w/v) agar. Sterile paper discs (0.25 in diameter; Becton Dickinson) were applied to the surface of the agar and the plates dried for 1 h at 37°C. Solutions of iron-saturated Tfs (4 mg/mL; 20-μL volumes) or $FeCl_3$ (1 mg/mL; 10-μL volumes) were added to the discs, as appropriate, and after drying for a further 1 h at 37°C, the plates were incubated for 24 h at 37°C in an atmosphere enriched with CO_2 (4-10%; BBL GasPak). Results were scored based on evident growth or no evident growth surrounding the discs.

2.3.4 Liquid cultures and preparation of membrane fractions

The Tryptone and yeast extract used in these experiments were each from a single batch. sTYE-H was used for growth of organisms under iron-replete conditions.

For growth under iron-replete conditions plus Tf, sTYE-H was further supplemented with bovine Tf (to 80 μg/mL). For growth under iron-restricted conditions, sTYE-H was supplemented routinely with EDDA (18 - 24 μM; added to the TYE-H prior to autoclaving) such that the final cell yields were approximately 80% of those obtained with iron-replete cultures. In one set of experiments, sTYE-H was supplemented with EDDA to 50 μM; depending on the strain, final cell yields were ≤ 10% of those obtained with iron-replete cultures. For growth under iron-restricted conditions in the presence of Tf, sTYE-H was supplemented with EDDA (to 50 μM) and with bovine Tf (as above). The iron contents of the TYE-H and TYE-H + 50μM EDDA were determined using a ferrozine-based assay (Sigma); the ferrozine-detectable iron contents were found to be 8.8 and 0.95 μM, respectively. Media (200-mL volumes) were contained in 1-L, acid-washed DeLong flasks fitted with Morton closures (Bellco Glass) or in 1-L screw-capped flasks (Nalgene). All cultures (1% (v/v) inoculum) were incubated at 37°C, with agitation on a gyratory shaker (200 rpm), in an atmosphere not enriched with CO_2. Growth was monitored turbidimetrically at 660 nm (Gilford Stasar II spectrophotometer); when grown in sTYE-H, all strains attained similar final turbidities (OD_{660} ~ 1.0). The organisms were harvested in stationary phase, washed and resuspended as described previously (Niven *et al.*, 1989) and used to prepare total membrane and outer membrane-enriched fractions essentially as described by Niven *et al.* (1989). For experiments with intact cells, the organisms were washed and resuspended as described above, or with Tris-buffered saline (TBS; 145 mM NaCl, 100 mM Tris-HCl, pH 7.4), such that the OD_{660} was 1.0.

2.3.5 Transferrin-binding assays

Transferrin-binding assays (dot blots) were carried out as described by Schryvers and Morris (1988a) but with some modifications. Briefly, total membranes (0.5 mg protein/mL; 50-µL volumes), or in some experiments, intact cells (buffered suspensions, as above; 50-µL volumes), were applied to nitrocellulose sheets (0.45 µm pore size; Schleicher and Schuell) held in a dot blot apparatus (Minifold I; Schleicher and Schuell), the liquids drawn through the nitrocellulose and the "dots" dried for 30 min at 37°C. Following removal from the apparatus, sheets were incubated for 1 h at 37°C, with gentle agitation, in TBS containing 0.15% (v/v) Tween 20 (TTBS). Sheets were then returned to the apparatus and an appropriate biotinylated Tf (500 ng in 250 µL TTBS), or HRP-conjugated Tf (~125 or 500 ng in 250 µL TTBS), added to each well; in competition experiments, native Tf (0.1 mg), as appropriate, was added with biotinylated ovine Tf. Samples were incubated for 1 h at 37°C, the Tf solutions removed and each well rinsed individually with 500 µL TBS. Sheets were then removed from the apparatus and following three 10-min washes with TBS (37°C, with gentle agitation), were either (HRP-conjugated Tfs) developed with the 4-chloro-1-naphthol/H_2O_2 reagent described by Niven *et al.* (1989) or (biotinylated Tfs) incubated for 1 h (as above) with streptavidin-HRP (Gibco BRL) diluted 1:1000 in TTBS and then washed and developed as described above.

2.3.6 Isolation of transferrin-binding polypeptides and SDS-PAGE

Tf-binding polypeptides were isolated from total membranes using biotinylated bovine Tf plus streptavidin-agarose (Gibco BRL) and the batch affinity procedure and wash system 3 developed by Schryvers and Morris (1988b) as modified by Ricard *et al.* (1991). To identify the isolated polypeptides, the washed affinity resin pellets were resuspended with 200 µL of a sample buffer containing 2% (w/v) SDS, 30% (w/v) glycerol and 0.1% (w/v) bromophenol blue in 200 mM Tris-HCl, pH 6.8, immersed in boiling water (5 min), cooled on ice and centrifuged (1000 × *g*, 3 min, 20°C). Supernatant fractions were transferred to clean tubes, 2-mercaptoethanol was added to 1.4 M and the samples incubated for 15 min at 20°C. Samples were mixed thoroughly and 100-µL volumes were subjected to SDS-PAGE using a Protean II electrophoresis system (Bio-Rad); the electrode buffer was 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS and the gels were prepared as described previously (Niven *et al.* 1989). Total membrane and outer membrane-enriched fractions were run concomitantly; such fractions were diluted with an equal volume of a sample buffer containing 4% SDS, 20% glycerol, 10% (v/v) 2-mercaptoethanol and 0.02% bromophenol blue in 125 mM Tris-HCl, pH 6.8, immersed in boiling water (5 min), cooled on ice and loaded (5 µg protein per lane). Low molecular weight protein standards (Amersham Pharmacia; 5.8 µg protein per lane) were treated similarly. Electrophoresis (10 mA per gel) was conducted until the tracking dye neared the bottom of the gel (~15 h). Separated polypeptides were visualized by silver staining using GelCode SilverSNAP (Pierce Chemical).

2.3.7 Estimations of protein concentrations

Protein concentrations were estimated by the method of Gornall *et al.* (1949) using bovine serum albumin (fraction V powder; Boehringer-Mannheim) as the standard.

2.3.8 Imaging

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

2.4 Results

2.4.1 Iron acquisition from transferrins

Plate assays were used to investigate the ability of *H. ovis* to acquire iron from a variety of animal Tfs. All five strains of *H. ovis* exhibited obvious growth around discs containing ovine, bovine and goat Tfs, but not around discs containing porcine or human Tfs (Table 2.1).

2.4.2 Transferrin binding assays

Total membranes derived from strains 9L and 642A that were grown under iron-restricted conditions in the presence or absence of bovine Tf demonstrated strong binding of biotinylated ovine, bovine and goat, but not porcine or human, Tfs (Fig. 2.1). Identical results were obtained with the other three strains (714, 5688T and 3384Y) but only if the total membranes were derived from organisms grown under iron-restricted

Table 2.1. Growth of *H. ovis* strains with different Tfs as iron sources[a]

Strain	oTf	bTf	gTf	pTf	hTf	$FeCl_3$
9L	+	+	+	-	-	+
642A	+	+	+	-	-	+
714	+	+	+	-	-	+
5688T	+	+	+	-	-	+
3384Y	+	+	+	-	-	+

[a]The sources of the Tfs were as follows: o = ovine, b = bovine, g = goat, p = porcine and h = human.

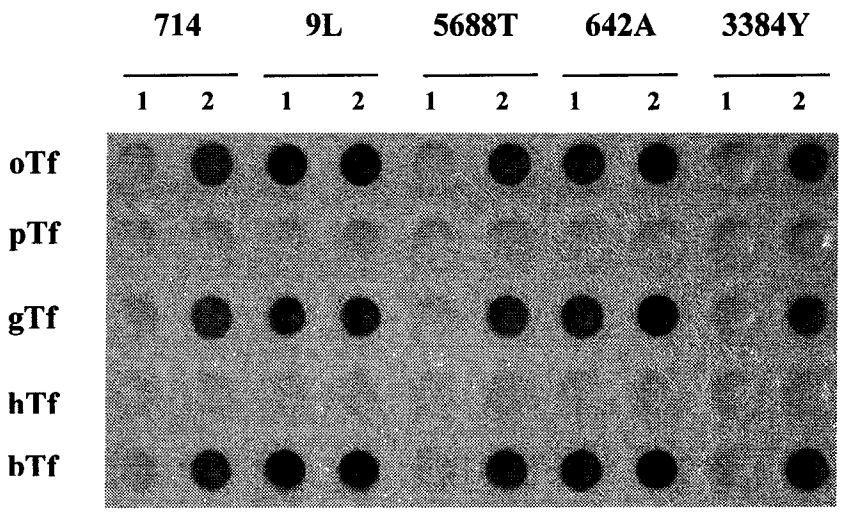

Fig. 2.1. Dot blot demonstrating binding of the indicated Tfs by total membranes from strains of *H. ovis* grown under iron-restricted conditions in the absence (1) or presence (2) of bovine Tf. The Tfs are abbreviated as follows: o = ovine, b = bovine, g = goat, p = porcine and h = human.

conditions in the presence of bovine Tf (Fig. 2.1). Similarly, when all five strains were grown under iron-restricted conditions in sTYE-H containing 50 μM EDDA (rather than 18-24 μM EDDA), binding of biotinylated bovine Tf was observed with intact cells of strains 9L and 642A, but with none of the other strains (results not shown). Binding of biotinylated Tfs (ovine, bovine and goat) was also observed with intact cells grown under iron-restricted conditions in the presence of ovine Tf (results not shown). With all five strains, total membranes derived from organisms grown under iron-replete conditions in the presence or absence of bovine Tf failed to bind any of the biotinylated Tfs. Also, in all experiments in which total membranes were used, identical results were obtained when HRP-conjugated Tfs were used in place of the biotinylated Tfs plus streptavidin-HRP (results not shown).

Competition experiments were performed to determine if the ovine, bovine and goat Tfs were being recognized and bound by the same receptor(s). The results presented in Fig. 2.2 were obtained using total membranes from organisms grown under iron-restricted conditions in the presence of bovine Tf since such conditions were associated with the binding of Tf by all five strains. Excess native ovine, bovine and goat, but not porcine or human, Tfs were able to block the binding of biotinylated ovine Tf (Fig. 2.2). Similar results were obtained with total membranes derived from strains 9L and 642A grown under iron-restricted conditions in the absence of Tf and also when HRP-conjugated ovine Tf was used in place of biotinylated ovine Tf plus streptavidin-HRP (results not shown).

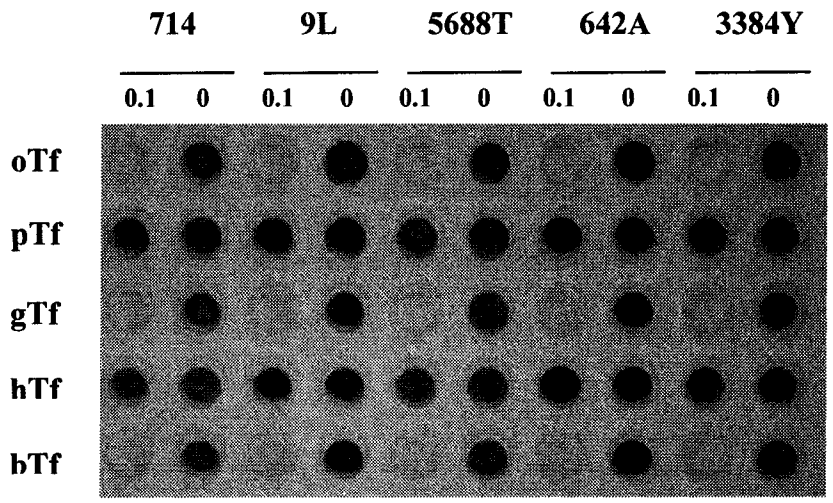

Fig. 2.2. Dot blot demonstrating competition between native Tfs and biotinylated ovine Tf for Tf binding sites on total membranes from strains of *H. ovis* grown under iron-restricted conditions in the presence of bovine Tf. The presence (0.1 (mg)) and absence (0) of competing Tfs are indicated. Competing Tfs are indicated on the left of the figure and are abbreviated as in Fig. 2.1.

2.4.3 Isolation and identification of transferrin-binding polypeptides

When grown under iron-replete conditions, the presence of bovine Tf in the growth medium had little, if any, effect on the total or outer membrane protein profiles obtained with any of the five strains (Figs. 2.3 through 2.7). On the other hand, when compared with membranes from organisms grown under iron-replete conditions, growth under iron-restricted conditions in the presence or absence of bovine Tf resulted in the appearance of one or more new polypeptides in the outer membranes of all strains (Figs. 2.3 through 2.7). To determine if any of these polypeptides represented Tf receptors, isolation of receptor components was attempted using total membranes and a modification (Ricard *et al.*, 1991) of an affinity procedure (Schryvers and Morris, 1988b) based on biotinylated bovine Tf plus streptavidin-agarose; biotinylated bovine Tf was used rather than biotinylated ovine Tf since bovine Tf is commercially available and it was apparent from the competition binding assays that ovine and bovine (and goat) Tfs are bound by the same receptor(s) (Fig. 2.2). Polypeptides with estimated molecular masses of 78 kDa and 66 kDa were isolated from all five strains grown under iron-restricted conditions in the presence of bovine Tf (Figs. 2.3 through 2.7). Although the 66-kDa Tf-binding polypeptide isolated from strain 642A is not clearly visible in this image (Fig. 2.4), such Tf-binding polypeptides have been isolated and visualized on several occasions (results not shown). Interestingly, when the organisms were grown under iron-restricted conditions in the absence of Tf, the 78- and 66-kDa polypeptides could be isolated from total membranes of strains 9L and 642A (Fig. 2.3 and 2.4) but not from membranes derived from the other three strains (Fig. 2.5 through 2.7). Collectively, these results are reminiscent of the results obtained in the Tf binding

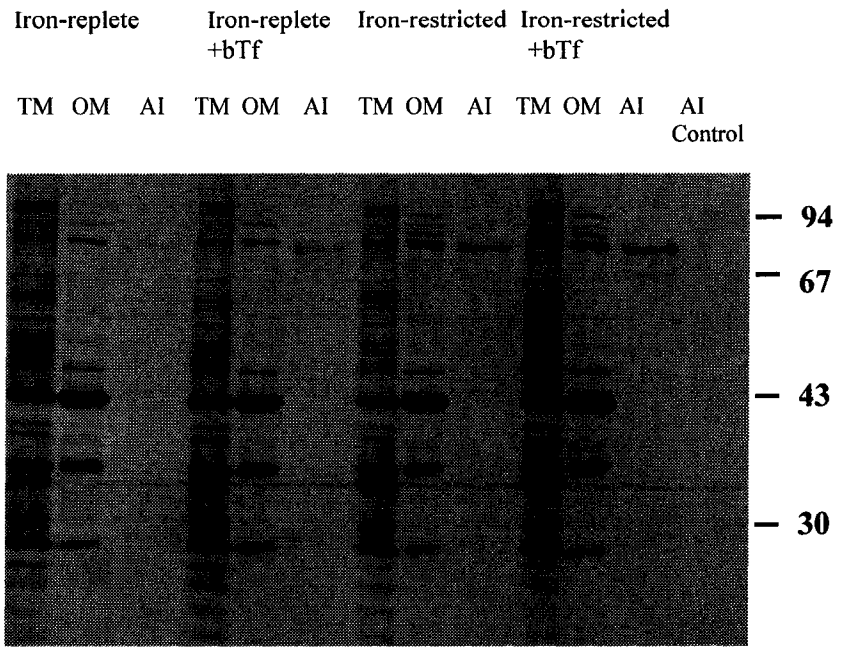

Fig. 2.3. SDS-PAGE of total membranes (TM), outer membranes (OM) and affinity-isolated polypeptides (AI) from *H. ovis* strain 9L grown under the indicated conditions. AI Control refers to a control sample obtained when the affinity isolation procedure was performed in the absence of biotinylated Tf. The numbers refer to the sizes (kDa) and positions of protein standards.

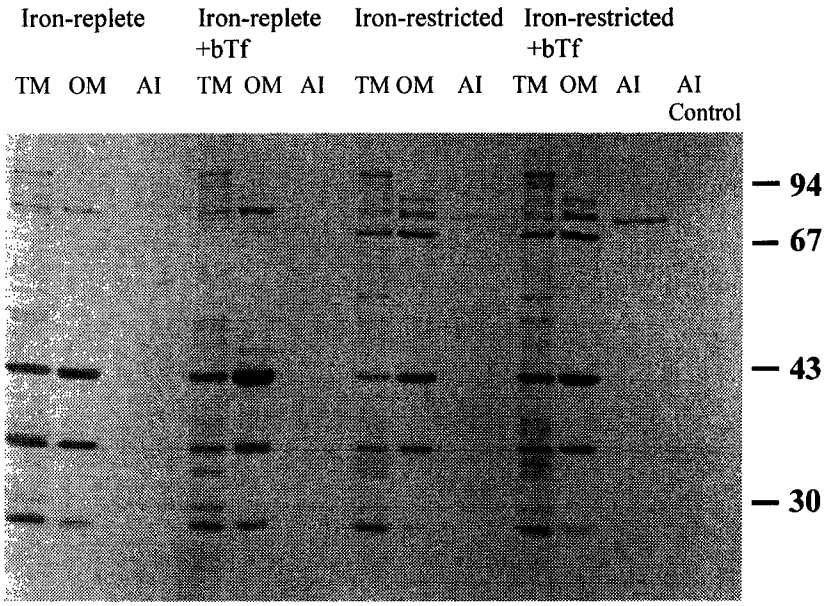

Fig. 2.4. SDS-PAGE of total membranes (TM), outer membranes (OM) and affinity-isolated polypeptides (AI) from *H. ovis* strain 642A grown under the indicated conditions. AI Control refers to a control sample obtained when the affinity isolation procedure was performed in the absence of biotinylated Tf. The numbers refer to the sizes (kDa) and positions of protein standards.

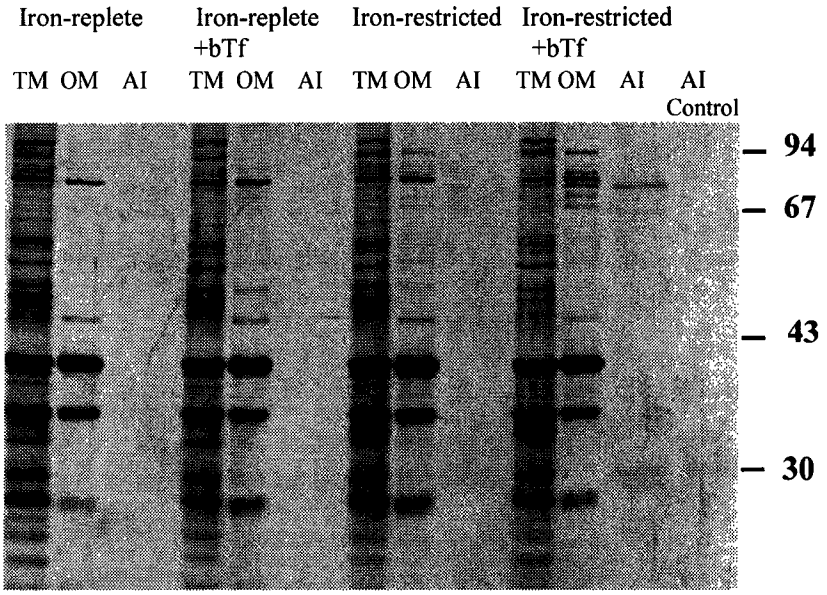

Fig. 2.5. SDS-PAGE of total membranes (TM), outer membranes (OM) and affinity-isolated polypeptides (AI) from *H. ovis* strain 3384Y grown under the indicated conditions. AI Control refers to a control sample obtained when the affinity isolation procedure was performed in the absence of biotinylated Tf. The numbers refer to the sizes (kDa) and positions of protein standards.

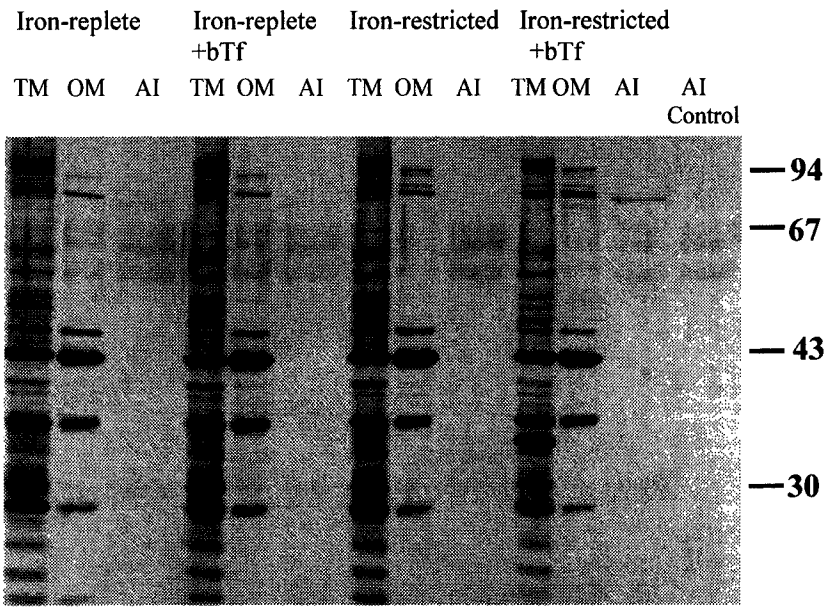

Fig. 2.6. SDS-PAGE of total membranes (TM), outer membranes (OM) and affinity-isolated polypeptides (AI) from *H. ovis* strain 5688T grown under the indicated conditions. AI Control refers to a control sample obtained when the affinity isolation procedure was performed in the absence of biotinylated Tf. The numbers refer to the sizes (kDa) and positions of protein standards.

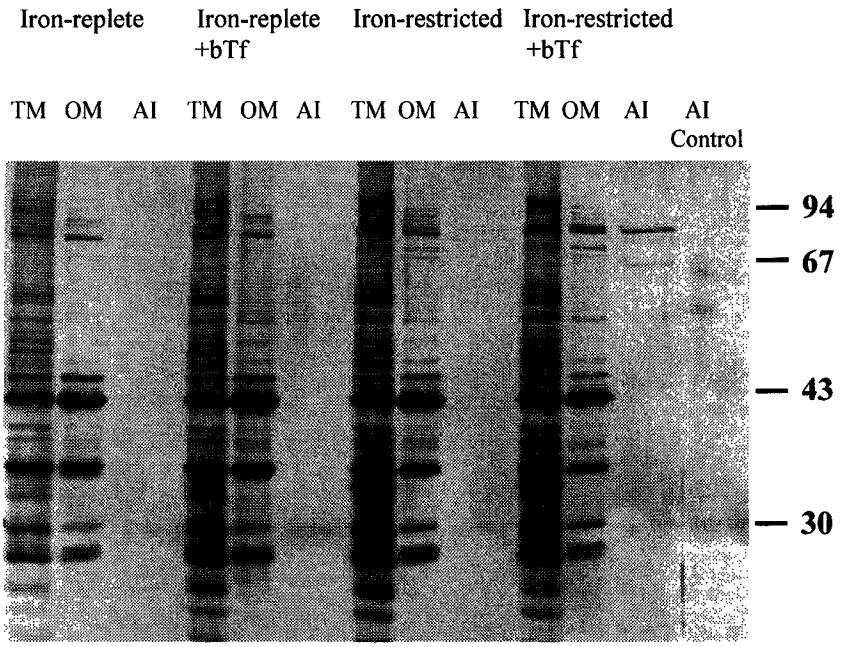

Fig. 2.7. SDS-PAGE of total membranes (TM), outer membranes (OM) and affinity-isolated polypeptides (AI) from *H. ovis* strain 714 grown under the indicated conditions. AI Control refers to a control sample obtained when the affinity isolation procedure was performed in the absence of biotinylated Tf. The numbers refer to the sizes (kDa) and positions of protein standards.

assays (Fig. 2.1). While trace amounts of the 78-kDa polypeptide were isolated from total membranes from strain 9L grown under iron-replete conditions in the presence of bovine Tf (Fig. 2.3), this was not observed on other occasions and except for this instance, Tf-binding polypeptides could not be isolated from total membranes of any strain grown under iron-replete conditions in the presence or absence of bovine Tf or from control samples where the biotinylated Tf was omitted (Figs. 2.3 through 2.7).

2.5 Discussion

The family *Pasteurellaceae* contains a variety of host-specific, iron-requiring pathogens of animals. Although *H. ovis* and *H. somnus* are closely related (Stephens *et al.*, 1983; Walker *et al.*, 1985), there appear to be differences in the capacities of these organisms to cause disease in sheep and cattle (see e.g., Biberstein, 1981; Lees *et al.*, 1994; Young and Hoerlein, 1970) and it has been hinted that such differences might be related to the Tf specificities of the organisms (Lees *et al.*, 1994). In the present study, we have demonstrated that unlike *H. somnus*, which can obtain iron only from bovine Tf (Yu *et al.*, 1992), all five strains of *H. ovis* resembled *M. haemolytica* (Yu *et al.*, 1992) in that they were capable of using ovine, bovine and goat, but not porcine or human, Tfs as iron sources for growth. Depending on the growth conditions, all five strains of *H. ovis* also resembled *H. agni* (Yu and Schryvers, 1994) (and *P. haemolytica*; Yu *et al.*, 1992) and now *P. multocida* (Ogunnariwo and Schryvers, 2001) in that total membranes were capable of binding all three (ovine, bovine and goat) Tfs specifically (Fig. 2.1), and apparently, by means of the same receptor(s) (Fig. 2.2). In effect, it has been established that with respect to Tf specificities, *H. ovis* (and *H. agni*) and *H.*

somnus are quite different and it would begin to appear that Tf specificity may, indeed, be a determinant of host specificity.

The Tf specificities of the *H. ovis* strains (Table 2.1 and Fig. 2.1) indicated that the acquisition of Tf-bound iron did not involve siderophores and suggested the existence of receptor proteins analogous to the Tbps of other organisms. However, the requirement of three strains (714, 5688T and 3384Y) for the presence of Tf, in addition to iron restriction, for the expression of Tf-binding activity (Fig. 2.1) was somewhat disconcerting in that such an effect had not been reported for any other organism. On the other hand, while all five strains produced one or more new outer membrane proteins in response to growth under iron-restricted conditions, irrespective of the presence of Tf in the growth medium, and while Tf-binding polypeptides could be affinity-isolated from total membranes of strains 9L and 642A grown under iron-restricted conditions in the presence or absence of Tf, strains 714, 5688T and 3384Y, in keeping with the results of the binding assays (Fig. 2.1), yielded Tf-binding polypeptides only when the membranes were from organisms grown under iron-restricted conditions plus Tf (Figs. 2.3 through 2.7). In all cases, the Tf-binding polypeptides had estimated molecular masses of 78 and 66 kDa and except for one occasion with strain 9L, they could not be isolated from membranes derived from organisms grown under iron-replete conditions, even if the medium contained Tf (Figs. 2.3 through 2.7). Typically, TbpA proteins have molecular masses of approximately 100 kDa but note that the presence of such proteins in affinity-isolated materials from at least two strains of *H. somnus* (Ogunnariwo *et al.*, 1990), and now *P. multocida* (Schryvers and Ogunnariwo, 2001) and *H. ovis* (Figs. 2.3 through 2.7), is not obvious.

Although the methods required for the isolation of the 78- and 66-kDa polypeptides indicate that these polypeptides do represent Tf-binding proteins, additional information that would help to identify either or both of these proteins as "classical" Tbps has yet to be obtained. For instance, following SDS-PAGE and transfer to nitrocellulose, neither the 78- nor 66-kDa polypeptides from strain 9L, unlike typical TbpB proteins (Gonzalez *et al.*, 1990; Griffiths *et al.*, 1990; Myers *et al.*, 1998; Schryvers, 1989; Schryvers and Lee, 1988; Schryvers and Morris, 1988a; Stevenson *et al.*, 1992), was able to bind biotinylated bovine Tf.

While the above results suggested that Tf had a role to play in the expression of the Tf-binding proteins of strains 714, 5688T and 3384Y, there was concern that expression was related to the EDDA contents of the media (18-24 μM for iron-restricted; 50 μM for iron-restricted plus Tf) rather than the presence of Tf. However, Tf-binding assays, using intact cells of all five strains grown in, and then concentrated from, medium containing 50 μM EDDA, demonstrated that this was not the case; Tf binding was exhibited by strains 9L and 642A but not by strains 714, 5688T and 3384Y (results not shown). In effect, the results presented in this chapter indicate that while iron restriction alone is sufficient to promote the expression of Tf-binding proteins by strains 9L and 642A, it would appear that the production of such proteins by strains 714, 5688T and 3384Y does require two signals, namely, iron restriction and the presence of Tf.

While the requirement for Tf (plus iron restriction) for the production of Tf-binding proteins by some strains of *H. ovis* is quite novel in that a specific, host-derived molecule is involved in expression, Fur plus a positive regulator are known to be

involved in the regulation of other iron uptake systems. For example, in *E. coli*, the binding of ferric citrate to the ferric citrate receptor (FecA) in the outer membrane induces transcription of the *fecABCDE* transport genes when iron is limiting (see e.g., Angerer and Braun, 1998; Braun, 1997; Enz *et al.*, 2000; Welz and Braun, 1998). Conceivably, a regulatory system analogous to the one above could be involved in the Tf-dependent regulation of receptor expression in *H. ovis*.

Chapter 3. Identification and sequence of *tbpA*, *fur* and *fldA* homologues in *H. ovis*

Part of the information presented in this chapter was adapted from the following:

Ekins, A., and Niven, D.F. 2002. Identification of *fur* and *fldA* homologs and a *Pasteurella multocida tbpA* homolog in *Histophilus ovis* and effects of iron availability on their transcription. J. Bacteriol. **184:** 2539-2542.

Figure 3.2 is reproduced with permission.

3.1 Summary

tbpA, *fur* and *fldA* homologues from two strains (9L and 3384Y) of *H. ovis* were sequenced. The predicted TbpAs of these strains are homologues of the *P. multocida* TbpA and collectively, represent the second example of a new subfamily of TonB-dependent receptors. The *fldA* homologue was found to be immediately upstream of *fur* in both strains and the predicted products of both of these genes were homologous to similar proteins found in *P. multocida*.

3.2 Introduction

It was demonstrated previously that the five strains (9L, 642A, 714, 5688T and 3384Y) of *H. ovis* are capable of binding, and removing iron from, specifically, ovine, bovine and goat, but not human or porcine, Tfs (Chapter 2; Ekins and Niven, 2001). Affinity isolation procedures allowed the isolation and identification of a major Tf-

binding polypeptide of 78 kDa, and a minor one of 66 kDa, from all five strains. Such Tf-binding polypeptides could be isolated from total membranes derived from two strains (9L and 642A) grown under iron-restricted conditions alone, whereas Tf-binding polypeptides could be isolated from total membranes derived from the other three strains (714, 5688T and 3384Y) only if the organisms were grown under iron-restricted conditions in the presence of Tf (Chapter 2; Ekins and Niven, 2001). The majority of the Tf-binding proteins (Tbps) characterized to date are expressed when the respective organisms are grown under conditions of iron-restriction (see e.g., Schryvers and Morris, 1988a; Ogunnariwo and Schryvers, 1990; Ogunnariwo *et al.*, 1990; Ricard *et al.*, 1991; Ogunnariwo and Schryvers, 2001); such regulation is due presumably, to the activities of ferric uptake regulator (Fur) proteins, which in the presence of iron, would repress the transcription of *tbp* genes (Gray-Owen and Schryvers, 1996). The objectives of this portion of my project were to identify and sequence the *tbp* genes, including upstream sequences, from two representative strains of *H. ovis* to determine their relationship to other characterized *tbp*s and to investigate the possibility that upstream sequences might provide some clue to the mechanism of Tf-dependent regulation of TbpA expression. Furthermore, since it was apparent that iron also plays a role in the expression of Tbps, the possibility of *H. ovis* possessing a *fur* homologue was examined.

3.3 Materials and methods

3.3.1 Organisms and storage conditions

E. coli strains DH5α and H1717 were grown in the tryptone-yeast extract (TY) broth described by Stojiljkovic *et al.* (1994) and were stored as described above (section 2.2.1). TY was solidified by the addition of 1.6% (w/v) agar and when necessary, was supplemented, as appropriate, with ampicillin, isopropyl-β-D-thiogalactopyranoside, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

3.3.2 N-terminal sequencing of the 78-kDa Tf-binding polypeptide

Tf-binding polypeptides were affinity-isolated from total membranes derived from *H. ovis* strain 9L grown under conditions of iron-restriction plus bovine Tf as described above (section 2.2.6) except that the washed affinity resin pellets were resuspended with 2% (w/v) SDS in 200 mM Tris-HCl, pH 6.8 prior to boiling. The proteins in the supernatant fractions from 10 affinity isolations were concentrated using Ultrafree-0.5 Biomax-30 (30 kDa MW cut-off) centrifugal filter devices (Millipore) as described by the manufacturer and washed with 100 mM NaCl, 50 mM Tris-HCl, pH 8.0 (400 μL). The retentates (~60 μL total) were pooled and frozen at -20°C. Affinity-isolated material was subjected to SDS-PAGE as described above (section 2.2.6) except that the electrode buffer in the upper reservoir also contained 100 μM thioglycolate. Samples were transferred to PVDF (Bio-Rad)(50 V; 24 h) using the buffer system described by Cornelissen *et al.* (1992). Transferred polypeptides were visualized by staining with Coomassie Brilliant Blue R-250 as described by manufacturer of the PVDF membrane (Bio-Rad) and the 78-kDa Tf-binding polypeptide was cut from the

membrane using a scalpel. The material, stored in 50% (v/v) methanol, was submitted to the Sheldon Biotechnology Centre (McGill University) where N-terminal analyses were performed in duplicate.

3.3.3 FURTA

Fur titration assays were preformed as described by Stojiljkovic *et al.* (1994; see section 1.4.3). Briefly, DNA isolated from *H. ovis* strains using a DNeasy Tissue Kit (Qiagen) was partially digested with *Sau*3AI and such DNA fragments were ligated into *Bam*HI-digested pUC19 or pBluescriptKS$^+$. The ligation products were used to transform *E. coli* H1717 and transformants were selected for on MacConkey agar plates supplemented with 25 μg/mL ampicillin and 40 μM $Fe(NH_4)_2(SO_4)_2$ and incubated for up to 48 h at 37°C. Plasmids were isolated from FURTA positive transformants using the QIAprep Spin Miniprep Kit (Qiagen) and inserts were sequenced using either pUC or T3 and T7 sequencing primers, as appropriate, and a BigDye sequencing kit (PE Biosystems). Sequence analyses were performed by the Applied Biotechnology Laboratory (Macdonald Campus, McGill University).

3.3.4 Identification and sequencing of *tbpA*, *fur* and *fldA* homologues

DNA was isolated from *H. ovis* strains using a DNeasy Tissue Kit (Qiagen) or Genomic-tip 20/G columns and the recommended buffer system (Qiagen). Alignment of the deduced amino acid sequences of the TbpAs from *P. multocida* (accession no. AY007725), *Pasteurella trehalosi* (accession no. AF312919) and *M. haemolytica* (accession no. U73302) using Clustal W (Biology Workbench;

http://workbench.sdsc.edu/) revealed at least three regions with homologous amino acids. The nucleotide sequence of *P. multocida tbpA*, corresponding to the conserved regions AIRGVDK and SKTGYTSKN, was used to design forward (PmF1, 5' GCTATCCGTGGCGTTGATAAA) and reverse (PmR2, 5' GTTTTTTGAAGTATAGCCGGTTTTAGA) primers, respectively, and using these primers, DNA from the five strains of *H. ovis* and the Expand High Fidelity PCR System (Roche Diagnostics), an appropriately-sized (~300 bp) fragment was amplified from each strain. These amplification products were ligated into pGEM-T Easy (Promega) and transformed into *E. coli* DH5α. Plasmids harboring the correct insert, as verified by colony PCR, were isolated from transformants and such inserts were sequenced using pUC sequencing primers and a BigDye sequencing kit. The sequence of the ~300-bp products obtained with the DNA from all five strains were very similar (results not shown) and a BlastX search revealed that all were homologous to the TbpA of *P. multocida*. The single-stranded sequence of *tbpA* from strain 9L was determined by sequencing products generated by inverse PCR (Ochman *et al.*, 1988) and (or) by direct genomic sequencing (Heiner *et al.*, 1998). Primers based on this single-stranded sequence were used to amplify *tbpA*, including upstream and downstream regions, from strains 9L and 3384Y using the Expand High Fidelity PCR System (Roche Diagnostics). The resulting PCR products, purified using the QIAquick PCR Purification Kit (Qiagen), were used directly as template for sequencing of both strands using primers based on the single-stranded sequence of *tbpA* of strain 9L. The nucleotide sequences of *tbpA* from strains 9L and 3384Y were submitted to GenBank and assigned accession numbers AY040784 and AY040785, respectively.

The conserved amino acid sequences VGLKITEPR and HHDHIICEDC in the *H. influenzae* Fur protein (accession no. U32704), as identified by Daniel *et al.* (1999), were used to design degenerate forward (Fur3, 5' GTIGGIYTIAARATIACIGARCCIMG) and reverse (Fur5, 5' RCARTCYTCRCARATRATRTGRTCRTGRTG) primers, respectively. Using these primers and DNA isolated from strain 9L, PCR yielded a product with the anticipated size of ~250 bp. This product was cloned and sequenced as above, and BlastX searches revealed that it encoded a Fur homologue. A strategy similar to that used for the sequencing of *tbpA* was employed to determine the single-stranded sequence of *fur*, and also *fldA*, from strain 9L and subsequently, the complete double stranded sequences of *fur* and *fldA* from strains 9L and 3384Y. The sequences of *fldA-fur* from strains 9L and 3384Y were submitted to GenBank and assigned accession numbers AF386645 and AF386646, respectively.

3.4 Results and discussion

3.4.1 Identification and sequencing of a *fur* and *fldA* homologues

The availability of iron influences the expression of Tbps in *H. ovis* (Chapter 2; Ekins and Niven, 2001) suggesting that *H. ovis* may possess a Fur homologue. Using the primers Fur3 and Fur5, a portion of the *fur* homologue from strain 9L was isolated and sequenced. While determining the single stranded sequence of the *fur* gene and its upstream and downstream regions, it was noted that immediately upstream (16 bp) of *fur*, there was an *orf* (*fldA*) encoding a flavodoxin homologue (Fig. 3.1). Since the intergenic region lacked an obvious promoter sequence, *fldA* and 190 bp upstream of

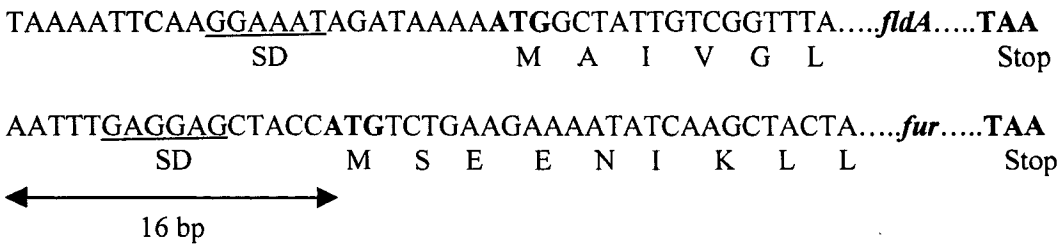

Fig. 3.1. Genetic organization of the *fldA-fur* genes in strains 9L and 3384Y. Putative SD sequences are indicated by underline and start and stop codons are indicated in bold.

fldA were sequenced to determine if either of these regions contained a promoter, but no putative promoter elements were found. The genetic arrangement, *fldA-fur*, has been observed in many organisms (see e.g. Zheng *et al.*, 1999), including *P. multocida* (May *et al.*, 2001). It has been suggested that flavodoxin maintains the reduced state of enzymatic Fe-S clusters and is involved in defense against oxidative stress (Zheng *et al.*, 1999). A well-documented association exists between oxidative stress defenses and the regulation of iron uptake (for a review, see Touati, 2000) suggesting that the genetic organization, *fldA-fur*, reflects a need for their coordinated expression (Zheng *et al.*, 1999). Although the arrangement of *fldA-fur* in *H. ovis* and the apparent lack of an obvious promoter immediately upstream of *fur* suggested that these two genes may be co-transcribed, this hypothesis remained to be verified experimentally (see Chapter 4). The 1200-bp nucleotide sequence encompassing the *fldA-fur* region from both strains are nearly identical (see GenBank accessions); the sequences differ only in the bases at positions 1086, within the coding region of *fur*, and 1177, immediately after the stop codon of the *fur* gene. BlastP searches revealed that the predicted *H. ovis* FldA and Fur proteins share 84 and 91% identity, respectively, with similar proteins found in the *P. multocida* PM70 genome (May *et al.*, 2001). Note, however, that *P. multocida* PM70 is an avian isolate, and that based on the genome sequence, this organism appears to lack a TbpA homologue. In support of this observation, Ogunnariwo *et al.* (1991) have reported that while bovine isolates of *P. multocida* are capable of acquiring iron from, specifically, bovine Tf, avian isolates of *P. multocida* are unable to acquire iron from either of the avian Tf species (chicken and turkey) tested.

3.4.2 Identification and sequencing of a *tbpA* homologue

It was apparent from previous work (Chapter 2; Ekins and Niven, 2001) that the Tf-binding polypeptides of *H. ovis* are produced under conditions of iron limitation due, presumably, to the activities of the Fur protein. Since the FURTA can be used to identify sequences containing a Fur box or a close match, and consequently iron-regulated genes, this technique was used in an attempt to identify putative *tbp* genes. Unfortunately, putative *tbp* genes were not identified but partial sequencing of the inserts in a number of FURTA positive transformants allowed the identification of several other, putative, iron-regulated genes (Table 3.1; Appendix 1). In addition to the FURTA, alternative approaches including PCRs using a variety of different primer combinations were employed in an effort to identify the *tbp* genes in *H. ovis*. The N-terminal amino acid sequence of the affinity isolated 78-kDa Tf-binding polypeptide from strain 9L (DSNPATTVPN) was determined but the identities of only the first three to five amino acids were considered to be definitive. Degenerate oligonucleotide primers based on this amino acid sequence and on sequences conserved in a variety of TbpAs, and also primers 223 and 224 of Ogunnariwo and Schryvers (1996), were used in PCRs with strain 9L DNA but amplification products of the appropriate size and (or) sequence were not obtained. For instance, in PCRs using primers 223 and 224, products of the appropriate size (~300 bp) were isolated consistently but DNA sequencing revealed that such products encode the partial sequence of a glutamate ammonia ligase homologue (results not shown).

Table 3.1. Characterization of inserts from FURTA positive transformants

Plasmid[a]	DNA source[b]	Size of insert	Primer used	% Shared identity with representative BlastX search results
pF1-A	9L	~750 bp	pUCF	81% (125/154)[c] with ClpB from *P. multocida* (AE006206)[d]
			pUCR	78% (128/164) with ClpB from *P. multocida* (AE006206)
p9L-3	9L	~1 kb	T3	47% (128/164) with PurF from *H. influenzae* (U32800)
			T7	64% (48/74) with conserved hypothetical protein from *H. influenzae* (U32802)
p9L-12	9L	~8 kb	T3	89% (116/130) with LipA from *P. multocida* (AE006230)
			T7	68% (51/75) with hypothetical protein from *S. enterica* ssp. *enterica* serovar *typhi* (AC627280)
p714-8	714	~400 bp	T3	84% (69/82) with MenC from *P. multocida* (AE006150)
			T7	84% (70/83) with MenC from *P. multocida* (AE006150)
p714-9	714	~600 bp	T3	48% (22/45) and 41% (12/29) with transcriptional regulator of AraC/Xyls family from *S. typhimurium* LT2 (AE008698)
			T7	82% (51/62) with unknown protein from *P. multocida* (AE006126) and 38% (21/55) and 46% (13/28) with transcriptional regulator of AraC/Xyls family from *S. typhimurium* LT2 (AE008698)

[a] All plasmids were derived from pBluescriptKS$^+$, with the exception of pF1-A that was derived from pUC19.
[b] Strain which was the source of DNA tested in the FURTA.
[c] Number of identical residues out of the total number aligned.
[d] GenBank accession number of the representative BlastX result.

Based on this experience, a subsequent publication by Ogunnariwo and Schryvers (2001), describing a novel TbpA from *P. multocida*, was found to be extremely interesting in that they reported having similar difficulties amplifying fragments of *P. multocida tbpA* using a rapid PCR-based approach (Ogunnariwo and Schryvers, 1996). The authors speculated that the failure to amplify appropriately-sized fragments may be due to a lack of "signature sequences" in the *P. multocida tbp* genes. Primers based on the *P. multocida tbpA* and DNA isolated from all five strains of *H. ovis* were then used in PCRs, which allowed the isolation and identification of a ~300-bp fragment from each strain that, based on BlastX searches, is homologous to the TbpA of *P. multocida*. Using this information, the complete double-stranded sequence of the *H. ovis* strains 9L and 3384Y *tbpA* homologues, including upstream and downstream regions, was determined. The deduced amino acid sequences of the mature TbpAs from strains 9L (733 amino acids) and 3384Y (734 amino acids) indicate molecular masses of approximately 83 kDa and the predicted proteins share 97% identity based on BlastP analyses. Using the web-based SignalP V2.0 (http://www.cbs.dtu.dk/; Nielson et al, 1997), the cleavage site, resulting in mature TbpAs from both strains, is predicted to be between residues 26 and 27 (ALSLA↓DSNPA). This prediction is in keeping with the results of the N-terminal sequence analysis since the first five amino acids of the affinity-isolated, 78-kDa Tf-binding polypeptide are identical to those following the predicted cleavage site. Furthermore, this confirms that the 78-kDa Tf-binding polypeptide isolated from strain 9L (and, most probably, 3384Y) is TbpA. The first two BlastP results revealed that the *H. ovis* TbpAs share 72-73% identity with the TbpA of *P. multocida* and 31% with a

haemoglobin receptor of *N. meningitidis*. As was the case with the TbpA of *P. multocida* (Ogunnariwo and Schryvers, 2001), the TbpAs of *H. ovis* share limited identity with "classical" TbpAs of other comparable organisms and such findings suggest that the *H. ovis* TbpAs represent, collectively, the second example of a new subfamily (Ogunnariwo and Schryvers, 2001) of TonB-dependent receptors. In effect, in addition to having established that the Tf specificities of *H. ovis* and *H. somnus* are quite different, it would appear that these two organisms employ related, yet distinct systems for acquiring Tf-bound iron.

Based on the requirements for the expression of the TbpAs of strains 9L and 3384Y, it was anticipated that there would be a difference in the promoter regions of the two genes; however, they were found to be identical (Fig. 3.2). The putative -10 and -35 sequences are identical to consensus σ^{70} RNA polymerase binding sites (see e.g. Wösten, 1998) and a potential Fur box (13 of 19 bases match the *E. coli* consensus; Escolar *et al.*, 1999) overlaps the -10 region. Following the ribosomal binding site is the uncommon start codon, TTG, which, in the *E. coli* genome, constitutes only 1.1% of start codons (Hannenhalli, 1999). Downstream of *tbpA* are two inverted repeats (Fig. 3.2) that may be involved in transcriptional termination and on the opposite strand, there is a partial *orf* encoding the carboxy-terminal end of a diacylglycerol kinase-like protein (see GenBank accession numbers). BlastX searches with sequence upstream of both *tbpA*s, including an additional 650 bases of single-stranded sequence (strain 9L; results not shown), failed to reveal any *orf* with significant homology to any protein in the database. While an involvement of Fur remains to be confirmed experimentally, the

TTAAAAAACTTGACAGTTAGGGCATTTATAATTATAAGAACCATTCTCATTTCTAT
-35 -10 Fur Box

TATATTTTTTACAGGGAGATTTTCATTTTGAAATCTAAGTAT.....*tbpA*.....**TAG**TCT
SD M K S K Y Stop

ATTGACATCCGCTCCGCCTGAAGGCAGGGGAGGATGTCAACACG

Fig. 3.2. Genetic organization of the *tbpA* genes in strains 9L and 3384Y. Putative -35, -10 and SD sequences are indicated by underline, a potential Fur binding site is indicated in bold italics, start and stop codons are indicated in bold and inverted repeats, possibly involved in transcriptional termination, are indicated in underlined italics.

putative Fur boxes in the promoter regions upstream of *tbpA* and the identification of *fur* homologues in both strains help to explain the requirement for iron-restricted conditions for the transcription of *tbpA*. The requirement for conditions of iron-restriction and the presence of a suitable Tf in the growth medium for the expression of Tf-binding activity by strain 3384Y (Fig. 2.1; Ekins and Niven, 2001) implies that the regulation of expression in this strain is more complex than in strain 9L; however, the regulatory mechanism(s) resulting in Tf-dependent expression is not apparent based on the information presented in this chapter.

To date, all of the organisms that acquire iron from Tf by a contact-dependent mechanism, with the probable exception of *P. multocida* (Ogunnariwo and Schryvers, 2001), employ a two-receptor system comprised of the proteins TbpA and TbpB (see e.g. Gray-Owen and Schryvers, 1996). In most organisms, the genes encoding the Tbps are arranged in an operon, with *tbpB* preceding *tbpA*, and the promoter controlling the expression of both genes is located upstream of *tbpB* (see e.g. Gray-Owen *et al.*, 1995; Gonzalez *et al.*, 1995; Legrain *et al.*, 1993; Ogunnariwo *et al.*, 1997). Although a *tbpB* homologue did not appear to be present upstream of the *H. ovis tbpA*, the genetic arrangement, *tbpBA*, is not conserved in all organisms. For example, the *tbpA* of *M. catarrhalis* is upstream of *tbpB*; these genes are separated by an unknown *orf* and each of the three genes appears to have its own promoter (Myers *et al.*, 1998). Notably, recombinant *M. catarrhalis* TbpB is able to bind Tf after SDS-PAGE and transfer to PVDF (Myers *et al.*, 1998), a defining characteristic of the TbpBs described to date (see e.g., Gonzalez *et al.*, 1990; Schryvers and Morris, 1988a; Schryvers, 1989; Stevenson *et al.*, 1992). Following SDS-PAGE, however, and transfer to nitrocellulose, the 78- and

66-kDa Tf-binding polypeptides from strains 9L and 3384Y failed to bind bovine Tf (results not shown). Although it is tempting, therefore, to conclude that *H. ovis* does not possess a TbpB or *tbpB* homologue, we cannot preclude their existence absolutely; in effect, the significance of the 66-kDa Tf-binding polypeptide remains obscure.

Chapter 4. Effects of iron availability on the transcription of *tbpA* and *fldA-fur*

Part of the information presented in this chapter was adapted from the following:

Ekins, A., and Niven, D.F. 2002. Identification of *fur* and *fldA* homologs and a *Pasteurella multocida tbpA* homolog in *Histophilus ovis* and effects of iron availability on their transcription. J. Bacteriol. **184:** 2539-2542.

Figure 4.1 and Table 4.1 are reproduced with permission.

4.1 Summary

tbpA transcripts were readily detected by RT-PCR with RNA isolated from strain 9L grown under iron-restricted conditions in the presence or absence of bovine Tf. Although *tbpA*- specific transcripts could be detected in RNA samples from cells of strain 3384Y grown under iron-restricted conditions, depending on the primer pair, *tbpA* transcripts were detected by RT-PCR predominantly when the RNA was from cells grown under iron-restricted conditions in the presence of bovine Tf. The *fldA-fur* genes are transcribed as a single unit and the availability of iron, and the presence or absence of bovine Tf in the growth medium, had no apparent effect on the relative amounts of the *fldA-fur* transcripts.

4.2 Introduction

The promoter structures of the *H. ovis* 9L and 3384Y *tbpA* genes were essentially identical, suggesting that the transcription of these two genes should take place under similar environmental conditions. To determine if this was indeed the case, RT-PCRs were performed in order to determine what effects the iron availability or the presence or absence of bovine Tf in the growth medium had on the relative amounts of *tbpA*-specific transcripts. Furthermore, the genetic arrangement of *fldA-fur* and the apparent lack of putative promoter structures immediately upstream of *fur* suggested that these two genes were transcribed as an operon. Whether this is in fact the case was also investigated by performing RT-PCRs.

4.3 Materials and methods

4.3.1 Growth of organisms for RNA isolation

H. ovis strains 9L and 3384Y were grown in 200 ml of sTYE-H as described previously (Section 2.2.1; Ekins and Niven, 2001). When the OD_{660} was ~ 0.05, 25-ml aliquots were removed quickly to sterile 125-ml screw-cap Nalgene flasks, EDDA (to 50 μM for iron-restricted conditions) and bovine Tf (2 mg) were added as appropriate, and the flasks returned to the incubator. The organisms were harvested in exponential phase (OD_{660} ~ 0.3-0.75; depending on the strain and growth conditions), washed immediately with phosphate-buffered saline and resuspended in RNAlater RNA Stabilization Reagent as described by the manufacturer (Qiagen). RNA was isolated

using the RNeasy Mini Kit (Qiagen) and contaminating DNA was removed by two consecutive on-column treatments with RNase-Free DNase (Qiagen). RNA concentrations were determined spectrophotometrically (260 nm; Lambda 3B UV/VIS spectrophotometer, Perkin-Elmer).

4.3.2 RT-PCR of *tbpA* and *fldA-fur* transcripts

RT-PCR was performed using the OneStep RT-PCR Kit (Qiagen), as described by the manufacturer, with 1 ng of the appropriate RNA sample (or an aliquot of DNA, as a positive control) and primers as indicated in Fig. 4.1. Negative controls, in which RNA isolated from cells grown under all conditions served as template for PCRs with the different primer combinations (as described in Fig. 4.1), did not yield any detectable amplification products (results not shown).

4.4 Results and discussion

The genetic organization of *fldA-fur* suggested that these genes may be co-transcribed and in keeping with this suggestion, RT-PCR using a forward primer (FldA) inside *fldA* and a reverse primer (Fur) inside *fur* yielded a product of the appropriate size (Fig. 4.1). Using a forward primer based on single-stranded sequence of DNA (strain 9L; results not shown) that is farther upstream of *fldA* than the submitted GenBank sequence, in combination with the reverse Fur primer also resulted in an appropriately-sized amplification product (results not shown) suggesting that the promoter controlling the expression of these two genes is located upstream of *fldA* and beyond the submitted

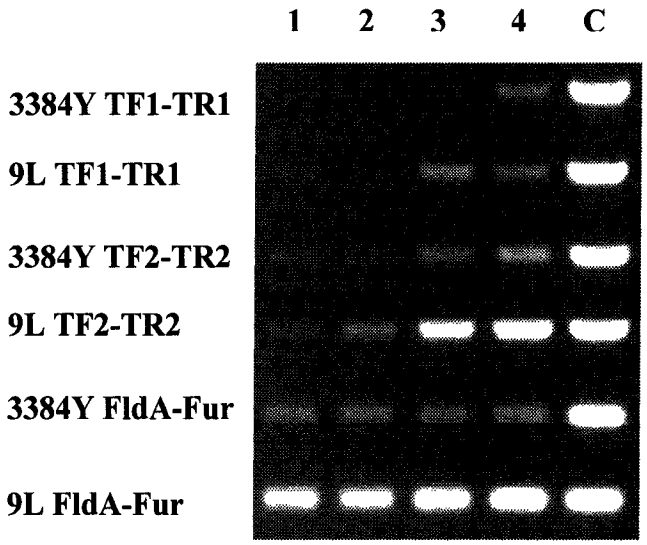

Fig. 4.1. RT-PCR using RNA isolated from cells grown under (1) iron-replete, (2) iron-replete + bovine Tf, (3) iron-restricted or (4) iron-restricted + bovine Tf conditions or (C) using total DNA. The strain from which the RNA (or DNA) was isolated and the primer pairs (described in Table 4.1) used are indicated along the left side of the figure.

Table 4.1. *tbpA*-, *fldA*-, and *fur*-specific primers

Primer	Description (Coordinates[a])	Sequence(5'→3')
TF1	*tbpA* Forward (432-453)	CCGTTGTGGCGAATGTTGAGCC
TR1	*tbpA* Reverse (1025-1004)	GCGGATTAGGTTTACCGCGATG
TF2	*tbpA* Forward (2065-2086)	AGTCAGCGGTGCTGTTAATGGC
TR2	*tbpA* Reverse (2556-2532)	GCGGCAAAATTACGTTTCGGTGCAG
FldA	*fldA* Forward (587-609)	GTTGATGACAATACATTCGTAGG
Fur	*fur* Reverse (930-907)	CCTCATCAAATTGGTTAAGTACAC

[a] Base pair coordinates given for TF1, TR1, TF2 and TR2 refer to the numbering given for *tbpA* of strain 9L (AY040784), while those given for FldA and Fur refer to the numbering given for *fldA-fur* of strains 9L and 3384Y (AF386645 and AF386646).

sequence. Although, it was not the intended purpose to determine if environmental conditions affected the transcription of *fldA-fur*, it was noted that the iron content of the medium and the presence or absence of Tf had no effect on the relative amount of the *fldA-fur* transcript. Since the amount of *fldA-fur* transcript, as detected by RT-PCR, remained essentially identical for each strain, regardless of the growth conditions, these transcripts could be used as controls to ensure that equivalent amounts of RNA, from organisms grown under the different conditions, were used in the RT-PCR experiments. While the TF1-TR1 primer pair used in RT-PCR with RNA isolated from strain 9L, grown under iron-restricted conditions in the presence or absence of bovine Tf, allowed the identification of *tbpA* transcripts, such transcripts were detected with RNA isolated from strain 3384Y only when the organisms were grown under conditions of iron-restriction in the presence of Tf (Fig. 4.1). Similarly, while TbpA was expressed by cells of strain 9L when grown under conditions of iron-restriction in the presence or absence of bovine Tf, TbpA was expressed by cells of strain 3384Y only if the organisms were grown under conditions of iron-restriction in the presence of bovine Tf (Fig. 2.1; Ekins and Niven, 2001). However, when the TF2-TR2 primer pair was used, RT-PCR allowed the identification of *tbpA* transcripts with RNA isolated from strains 9L and 3384Y grown under conditions of iron-restriction in the presence or absence of bovine Tf. Although RNA isolated from strain 3384Y, grown under conditions of iron-restriction, allowed the identification of *tbpA* transcripts, cells of 3384Y, which served as the RNA source, exhibited Tf-binding activity in a solid phase binding assay (section 2.3.5; Ekins and Niven, 2001) only if they were grown under iron-restricted conditions in the presence of bovine Tf (results not shown; see e.g., Fig. 2.1). The environmental

conditions required for TbpA expression in strain 9L are the same as those that resulted in the detection of increased amounts of *tbpA* transcripts by RT-PCR; on the other hand, with strain 3384Y, *tbpA* transcripts were detected readily by RT-PCR even when the growth conditions were such that TbpA expression was not detected (i.e. iron-restricted conditions). It would appear, therefore, that the expression of TbpA in strain 9L is regulated at the level of transcription, as is the case with other, comparable, organisms (Gray-Owen and Schryvers, 1996). The regulatory mechanisms controlling the expression of TbpA in strain 3384Y, however, appear to be more complex since appreciable amounts of *tbpA* transcript were detected (using primer pair TF2-TR2; Fig. 4.1) in RNA samples isolated from cells grown under iron-restricted conditions, conditions where Tf-binding activity could not be detected. This suggests that the regulation of TbpA expression in strain 3384Y may occur, to some extent, at the post-transcriptional level. One mechanism for post-transcriptional regulation involves the production of antisense RNA which is complementary to the mRNA from the gene it regulates; antisense RNA may act by inhibiting translation or possibly by destabilizing the mRNA (Chen and Crosa, 1996; Mizuno *et al.*, 1984).

Chapter 5: Tf-dependent expression of TbpA by *H. ovis* is due to phase variation

5.1 Summary

A poly G tract contained in the *H. ovis* strain 3384Y *tbpA* was suspected of being responsible for the Tf-dependent expression of TbpA. The region surrounding the poly G tract was amplified using DNA from *H. ovis* strains 9L and 3384Y grown in iron-replete media or iron-restricted media plus bovine Tf. Sequencing of the amplification products revealed that regardless of the growth conditions, the poly G tract in strain 9L contained 8 Gs, a situation that maintains the correct reading frame of the gene; similarly, the poly G tract in strain 3384Y contained 8 Gs when the organisms were grown under iron-restricted conditions in the presence of bovine Tf. However, sequencing of the amplification products from strain 3384Y grown under iron-replete conditions revealed that under these growth conditions the poly G tract contained 9 Gs, resulting in a frame shift and the introduction of a premature stop codon.

5.2 Introduction

Based on the results from RT-PCRs (Chapter 4), it was apparent that the mechanism(s) responsible for Tf-dependent expression of TbpA in *H. ovis* did not function solely at the level of transcription. During the assembly of sequence data pertaining to the *tbpA* gene of *H. ovis* strain 3384Y, problems were encountered while editing the sequence; there appeared to be a stretch of 9 Gs that would introduce a stop codon into the reading frame of the gene. Since 8 Gs were present at the comparable

location within the nucleotide sequence of the *H. ovis* strain 9L *tbpA* and since *H. ovis* strain 3384Y produced a 78-kDa Tf-binding polypeptide, it was assumed that the presence of an additional G in the *tbpA* gene of *H. ovis* strain 3384Y was due to a sequencing error. However, while writing the literature review for this thesis, it came to my attention that the expression of some haemoprotein receptors is affected by phase variation involving a poly G tract located within the coding region of the structural genes (Chen *et al.*, 1998; Lewis *et al.*, 1999; Richardson and Stojiljkovic, 1999). The aim of this part of the project was to determine if growth conditions have an effect on the nucleotide sequence of the poly G tract of the *tbpA* gene of *H. ovis* strain 3384Y.

5.3 Materials and methods

5.3.1 Growth of and harvesting organisms

Triplicate cultures of *H. ovis* strains 9L and 3384Y were grown overnight (~12 to 24 h, depending on the strain and growth conditions) in 25-ml volumes of sTYE-H (iron-replete) and sTYE-H supplemented with EDDA (to 50 μM) and bovine Tf (2 mg) (iron-restricted plus Tf) (as described previously; section 2.3.4); these media were contained in sterile 125-ml screw-cap Nalgene flasks. Aliquots (1 mL) were removed and were either supplemented with glycerol (to 15%) for storage at -80°C or harvested (16000 × *g*, 1 min), washed with 1 mL of 145 mM NaCl, harvested (as above) and resuspended in 10 mM HEPES, pH 7.4 (with KOH) such that the OD_{660} was 1.0 and stored at -20°C.

5.3.2 PCRs and DNA sequencing

1 µl of washed and resuspended cells were used directly in PCRs with primers TF1 and TR1 (Table 4.1). The amplification products were purified as described previously (see section 3.2.3) and used directly in sequencing reactions with TF1 or TR1 and the BigDye sequencing kit.

5.4 Results and discussion

Using cells, grown overnight in iron-replete medium or iron-restricted medium plus bovine Tf, as a source of DNA and primers TF1 and TR1, the regions encompassing the poly G tracts in *tbpA* from *H. ovis* strains 9L and 3384Y were amplified (see e.g Fig. 5.1) and sequenced. The sequences presented in Fig. 5.2 (e.g. 9L iron-replete) were determined by directly sequencing the poly G tract region amplified using three independent cultures and since both strands were sequenced, each sequence presented below is the result of at least 6 sequencing reactions. Regardless of the growth conditions, the *tbpA* gene from strain 9L invariably contained 8 Gs (Fig. 5.2) resulting in a reading frame that would allow the production of a full-length functional protein. While the poly G tract in the *tbpA* gene of strain 3384Y grown in iron-restricted medium plus bovine Tf contained 8 Gs, similar to the situation with strain 9L, the poly G tract in the *tbpA* gene of strain 3384Y grown in iron-replete medium contained 9 Gs which would result in a frame shift causing the introduction of a stop codon within the coding region (Fig. 5.2). Therefore, although elevated amounts of *tbpA*-specific transcripts are detected in RNA samples isolated from strain 3384Y grown under conditions of iron restriction (Fig. 4.1), a full-length functional TbpA would not be

Fig. 5.1. Approximate positions of primers TF1 and TR1 which flank the poly G tracts within the *tbpA*s of *H. ovis* strains 9L and 3384Y.

9L iron-replete

GGT GCC TTG GGG GGG GCT GTG GTG TTT ACC ACG AAA GAG ATT GAG
G A L G G A V V F T T K E I E

9L iron-restricted plus bovine Tf

GGT GCC TTG GGG GGG GCT GTG GTG TTT ACC ACG AAA GAG ATT GAG
G A L G G A V V F T T K E I E

3384Y iron-replete

↓
GGT GCC TTG GGG GGG GGC TGT GGT GTT TAC CAC GAA AGA GAT TGA G
G A L G G **G C G V Y H E R D Stop**

3384Y iron-restricted plus bovine Tf

GGT GCC TTG GGG GGG GCT GTG GTG TTT ACC ACG AAA GAG ATT GAG
G A L G G A V V F T T K E I E

Fig. 5.2. Nucleotide sequence surrounding the poly G tract (underlined) of *tbpA* amplified using DNA from *H. ovis* strains 9L and 3384Y grown in iron-replete medium or iron-restricted medium plus bovine Tf. An arrow indicates the putative position of the additional G in *tbpA* of *H. ovis* 3384Y when grown in iron-replete medium; the amino acids (and stop) resulting from the altered reading frame are indicated in bold.

translated from this transcript. By growing strain 3384Y (and possibly 5688T and 714) under iron-restricted conditions where the only source of iron is bovine Tf, a subpopulation of cells that contain 8 Gs in their *tbpA*, and therefore produce a functional TbpA, would most probably be selected for. Alternatively, as suggested by McKenzie and Rosenberg (2001), a selective environment may induce a state of transient hypermutation within a subpopulation of cells and a point mutation (9 Gs to 8 Gs) may be caused by the expression and activities of the error-prone DNA polymerase, DinB; *dinB* homologues have been identified in the genomes of *Actinobacillus*, *Neisseria* and *Pasteurella* spp. (McKenzie and Rosenberg, 2001).

In an attempt to assess the stability of the shift from 9 Gs to 8 Gs, glycerol-supplemented aliquots of strain 3384Y, grown under iron-restricted conditions in the presence of bovine Tf, were used to inoculate overnight cultures in iron-replete medium with the intention to test the hypothesis that in the absence of a selective pressure, cells grown initially in iron-restricted media with bovine Tf, would not revert to the 9 G genotype. This could be tested by growing the glycerol-supplemented aliquots of strain 3384Y in iron-replete medium followed by growth in iron-restricted medium; if the 8 G genotype is stable, strain 3384Y now grown in iron-restricted media should express Tf-binding activity that should be detectable using solid phase binding assays as described above. Unfortunately, it would appear that the glycerol-supplemented organisms (strain 3384Y) were not viable since growth was not detected after incubation for at least 48 h at 37°C.

Chapter 6. Conclusions and general discussion

All five strains of *H. ovis* are able to bind and acquire iron from, specifically, bovine, ovine and goat, but not human or porcine Tfs, suggesting that like other members of the *Pasteurellaceae*, *H. ovis* is capable of acquiring Tf-bound iron by means of a siderophore-independent receptor-mediated mechanism (Gray-Owen and Schryvers, 1996). Although *H. ovis* is thought to be closely related to *H. somnus*, which can acquire iron only from bovine Tf (Yu *et al.*, 1992), results presented in this thesis have demonstrated that with respect to Tf-binding specificities, *H. ovis* would appear to be more closely related to *M. haemolytica* (Yu *et al.*, 1992), *H. agni* (Yu and Schryvers, 1994) and *P. multocida* (Ogunnariwo and Schryvers, 2001), all of which are capable of binding the same spectrum of ruminant Tfs. The requirement for conditions of iron-restriction and the presence of a suitable Tf in the growth medium for expression of Tf-binding activity by three of the five strains of *H. ovis* is indeed unique and at the time, it was, to our knowledge, the only example of a human or animal pathogen apparently expressing a virulence determinant in response to a specific, host-derived molecule (see e.g., Mekalanos, 1992). Depending on the growth conditions, putative Tf-binding polypeptides of 78 and 66 kDa were affinity isolated from total membranes of all five strains. While this suggested the existence of a bipartite TbpA-TbpB receptor in *H. ovis*, the molecular masses of the Tf-binding polypeptides, and the failure of either of these polypeptides to bind bovine Tf after SDS-PAGE and transfer to nitrocellulose, suggested that these components did not represent "classical" Tbps.

The next logical step was to identify and sequence the Tbp structural genes, which was, as illustrated in section 3.4.2, much more difficult than originally anticipated. The report of Ogunnariwo and Schryvers (2001) describing a bovine isolate of *P. multocida* capable of binding ovine, bovine and goat Tfs, presumably through the activities of a single component TbpA protein whose structural gene did not contain *tbp* "signature sequences" suggested the existence of a comparable situation in *H. ovis*. Subsequently, primers based on the sequence of *P. multocida tbpA* allowed the identification and sequencing of *tbpA* homologues in two representative strains (9L and 3384Y) of *H. ovis*, and collectively, the products of these two genes represent the second example of a new subfamily of TonB-dependent receptors. Similar to the situation with *P. multocida*, *H. ovis* does not appear to produce a TbpB, or contain a *tbpB*, homologue, suggesting that TbpA can independently and effectively acquire Tf-bound iron; the significance of the 66-kDa Tf-binding polypeptide, however, remains unknown. Nonetheless, an organism acquiring Tf-bound iron in the absence of a TbpB homologue is not implausible since isogenic *tbpB* mutants are still capable of acquiring Tf-bound iron (Anderson *et al.*, 1994; Cornelissen *et al.*, 1992; Gray-Owen *et al.*, 1995; Luke and Campagnari, 1999).

Based on the requirements for the expression of the TbpAs of strains 9L and 3384Y, a difference in the promoter regions of the two genes was anticipated; however, they were found to be identical suggesting that despite their existence in different genetic backgrounds, these two genes should be transcribed under similar conditions. While *tbpA*-specific transcripts could be detected in RNA samples from strain 3384Y grown under iron-restricted conditions, similar to strain 9L, depending on the primer

pair used, *tbpA* transcripts were detected predominantly with RNA samples from strain 3384Y grown under iron-restricted conditions in the presence of bovine Tf. While putative Fur boxes located within the promoter region of the *H. ovis tbpA* genes and the identification of *fur* homologues in both strains help to explain the iron-repressible nature of Tf-binding activity, the mechanism of Tf-dependent expression of TbpA by strain 3384Y (and possibly 5688T and 714) did not appear to involve the promoter region suggesting the existence of a post-transcriptional regulatory mechanism. Somewhat serendipitously, while editing the sequence of *tbpA* of strain 3384Y, a stretch of 9 Gs, that would cause a frameshift resulting in the introduction of a premature stop codon, was encountered within the coding region. Although this result was considered to be an error at first, it became apparent that the expression of some haemoprotein receptors is affected by phase variation involving a poly G tract located within the coding regions of the structural genes (Chen *et al.*, 1998; Lewis *et al.*, 1999; Richardson and Stojiljkovic, 1999). The existence of 9 Gs within the *tbpA* of strain 3384Y when grown under iron-replete conditions was confirmed, suggesting that a mechanism analogous to the phase variation responsible for haemoprotein receptor expression may be responsible for Tf-dependent expression of TbpA in *H. ovis*. Questions relating to the stability of the shift from 9 Gs to 8 Gs and the possible existence of a specific mechanism(s) directing such a point mutation remain unanswered.

Appendix 1. Partial DNA sequences of FURTA positive clones

>pF1-A pUCF
CCAAACGCTTGCGACTTGCTTCATCATCTTCTTTTTGTAACGCTTGTTGTTCC
AATTTTAATTGAATAATACGGCGTTCTAATCTATCTAAAGGTTGTGGCTTAC
TATCAATTTCCATACGAATACTTGAGGCTGCCTCATCAATTAAATCAATAGC
TTTATCCGGTAATTGACGATCGGAAATATAACGATGAGAAAGGGTCGCTGC
CGCAACAATTGCCGGATCGGTAATTTGCACATGGTGATGAATTTCATACCGC
TCTTTTAACCCACGTAAAATGGCAATCGTATCTTCAACTGTCGGCTCACCAA
CAAATACCTTTTGGAAACGGCGTTCAAGTGCGGCATCTTTTTCAATATATTG
GCGATATTCATCTAAAGTTGTTGCACCTACCAATGCAGTTCACCACGAGCTA
ACTTGGCTTTTACAAATTCCCGCATCCATTGGCCCCGTCAGTTTTTTCCCGCA
CCTACCCATAAGTG

>pF1-A pUCR
TGTATCGGGCGTGATGAAGAAATTCGCCGTGCCATTCAGGTTTTACAACGTC
GTACAAAAAATAATCCAGTATTAATTGGGGAACCAGGTGTAGGTAAAACAG
CGATTGTGGAAGGATTGGCACAACGTATCGTAAACGGTGAAGTACCTGAAG
GCTTAAAAGGAAAACGTGTTCTTTCTCTAGATATGGGAGCGTTAATTGCGGG
AGCCAAATATCGTGGTGAATTTGAAGAGCGTTTAAAAGCGGTTCTCAAAGA
ATTGGCTCAAGAAGAAGGTCGTGTGATTTTATTTATTGATGAAATTCACACT
ATGGTAGGTGCGGGAAAAACTGACGGGGCAATGGATGCCGGGAAATTTGTT
AAAGCCAAGTTTAGCTCGTGGTGAACTGCATTGTGTAGGTGCAACACTTTAG
ATGAATATCCCCAATATATTGNAAAAGATGCCCCCTTTGACCCCCGTTTCCA
AAGGTTTTGTTTGGTGAGCCCACAGTTGAAAATCCATTGGCATTTTACNTGG
GTTTAAAANAGCGGG

>p9L-3 T3
GTTTTCAGTTGTCAATTGGATTTTTTTACCAGTCAACTCAATTTTCGATCCAC
TCGTATTTTCAACAGTATTAATCCCCTTCAAACTTGAATTCAAACTCAACGT
AACGCCTTTGCTATTCGTGTCAACCGACGTTGTGATGTTTTTATGGCTTCCGT
TTTCTTGTTTTCCTTTAATATGTAATTCATTATTCTTTTTATTTAATTCATAAC
CCGCTCCACTATCACCTTTAACAGTAAAGTTAGCAGCCACGTTTTCTAGTTG
TTTCTCAACATACTTTTTCGTTACAACACTATCATCGCTTTTCGGATCAAACA
ACACACCCTTTATCTACGCAGGATATTTTTGAGGCAGTTAAGAGCTACTCAC
AAAGATATTCGTGGTGCTTATGCTTGTGTTGCAATGATGATGGGTATGGATA
GTGCTTTCGTGATCTAGTGNGGTCTGTGGTGNGATCGGACCGCTACTCGACT
CGCTTTAATGACGGACTGACTTGAAAGGGAAAAAATCTGATGGCACGGTGA
ATGGAACGTTTAAACTACGTCACTTTGACAGA

>p9L-3 T7
TATCGCAGAAAAAGACGGCATTGTTGCGGCACAAACTAAATTACACCAACA
ACTGGAACAAAACCCAAGTACATTTATTTTACATCCGTTTTATTCAATACCA
AATTGATGCTGCTGAAAACGGCAAAGCAAAAGAAAGTTTGATTTTATTGCA
CAAAATCGTGGGAGATAGAATTGCACGAGGCTTTGATTATCGTTGCAGTCA
TTGCGGTTACCAAACACACAAACTATCATGGAATTGCCCATCTTGTCGGAAA
TGGGAAAAAATCAAACCGATTGTTGGAACTGAACACCACTAAAAAAGCAAC
TGTTTAAGAAAATTTCAATAGAGATGTGTTTTGAAAACCTTAGCAATCTTCA
GTTATTTTACGAATTTTGTTAGCGGATAAAATAATTTTCTGTGTGTTTTTTTC
AAATTTTTAAACTCAATAAACCCAAGTCGCTTATAAAATGAGACGCATGAT
CCGTTTGCTCGAATTATCTGTGCAAGAATGTGGAACGGATGATTTAAAACCA
TTACCATATCACCGATGCATCTGATTATTTCCTTTACAGGCTGATCCGACTTA
AAACC

>p9L-12 T3
GATGTAATAACGACATAACGTAATTTCATATCTTGAATGGTTTCCGCCAATT
TTTTTGGTTCATCCGGATCTGGGGGTAATGGTTTTCCATGGGCAACATCGCA
GAATGGACATCGGCGTGTACAAATTGCACCTAAAATCATAAATGTTGCTGT
GCCGTGATTGAAGCATTCATGTAAATTTGGACAAGACGCTTCTTCACATACT
GAATGTAATCCATGACGACGCATTCCGTTTTTAATACTGTTAATTTTCGCAG
AATTTGCAGGAAGCTTGATTTTCATCCATTCAGGTTTTTTTAATAACTCCTGA
TTTGGATCGATATTTTTAACCGGAATGATTGACGTTTTTGCGGCATCACGAT
ATTTGACACCACGTTCCATTTAAAAGGGGTTGCATTACAGACCTAATTTGTA
ATAAAGTTGTACATCATTATAGCCCAATAATTG

>p9L-12 T7
AGCTTATTTCGGAAATGAGTTGATTGAAAAAATTAATCCGACAAACGACGT
TAACAAACAAGGNAAATTTAATATGGATCAGCTTGTATCAGACTAATTTCTT
CTATTACGTTTTGATTAGCTGGAAAAAGTGCGGTAGGATTTTTCGTCATTTC
CGGCATGAGGATTATTTTGTGGCGTAAAGGTAAATTGAGTTCTAATTCTGCA
CTTAATCCGCCAATCATGAGCCACTGATCAATAGTAAAGGTTTTCTAATTGT
TTTACCTTTTTATGGGCAAATCATTTGGGACGTTCAATTTAACGGACAAACT
CTCACAATGAAAAATATGTTCAACGAACCTAAGCAGGGAACAACTATTATT
GATACCTATGGGTGTTTTGCTTTCCATTCAGGCTTAATTCGTAATGGTTGCCC
AAGTCCAAAAGACGAGCATCCTTTGCATGGTGAAATGCCTTGTGCCGAATG
GATAANGCTTG

>p714-8 T3
GGGAATTGCTTCTAACAACATATCAGCAATCATACCATCACGATTTGCTTCA
TACATGCCAACCTTAATTTTAGCTACCTTCTCCCCTGGAATTTGATTTAATAC
ATCATAAAGCTCATCAGGATCGCCGTAACATAACGGGGCGACTTGATAGTT
TCCTTCTTGCACCAATTCTCCTTCCATTTCAGCCAAAGCACAACTTAAGCCA
AAAGCAACAGACGGATATAGCCCTTGCAATGAAAATTTTTGATTTGCAGAT
CAAAGCGGCACCTGTGGAAAGACCGATGACCGTGACCAATATTAAAGACGG
GGATTTAACCGATAAGTCAACTGATGCGGTCAACGGTTCGCAATTGGTGAA
AGCCACGGGGGCGAAGTTTATTGATGATCC

>p714-8 T7
AATAAACTTCGCCCCCGTGGCTTTCACCAATTGCGAACCGTTGACCGCATCA
GTTGACTTATCGGTTAAATCCCCGTCTTTAATATTGGTCACGGTCATCGGTCT
TTCCACAGGTGCCGCTTTGATCTGCAAATCAAAAATTTTCATTGCAAGGGCT
ATATCCGTCTGTTGCTTTTGGCTTAAGTTGTGCTTTGGCTGAAATGGAAGGA
GAATTGGTGCAAGAAGGAAACTATCAAGTCGCCCCGTTATGTTACGGCGAT
CCTGATGAGCTTTATGATGTATTAAATCAAATTCCAGGGGAGAAGGTAGCT
AAAATTAAGGTTGGCATGTATGAAGCAAATCGTGATGGTATGATTGCTGAT
ATGTTGTTAGAAGCAATTCCCGAT

>p714-9 T3
TTGTCTAAATTTAACGTTGCTGTGTAAACATATATTGCAAATGTATAAAAAT
CTATCCTTACTTTTACTTGATGATTTGTTCGATTTAGAATTAGTTGCGGGTCT
TACTCAATGTGAGAAAGGTAATTACCTTGATTCTATGGTAGATAGACAACA
AGGAATGAGTGGCTATATGTTGCAATTAACTACTTTTGGTACAGGCATTATT
TTCGATGGTAAACATTCTTTTTCCCTCATCGAGGACAGCTGCTACTATTCAC
ACCAAGTGCGGTTCAACATTATCACAGACATCCAGAAAGTCAGTATTGGCA
CTATAAATGGATCTTCCTTGAGCTGTTGAGCTAAAGTAAAAAAACTGTCCCA
AGTTTCATTTGTGCACGATACTGCTCCAATTTGAGCGCCTCAAGAGAATGGG
TTA

>p714-9 T7
GTGGGAGAATATTGCATAGTGCGGCTTTCCGTTGTTTACAAGCAAAAACAC
AAATTCACGCTATAGGCGAAAATGATTTTTACCGCACTCGTTTAACCCATTC
TCTTGAGGTCGCTCAAATTGGTAGCAGTATCGTTGCACAAATGAAACTTGTG
GACAGTTTTTTTACTTTAGCTCAACAGCTCAAGGGAAGATCCATTTATAGTG
CCAATACTGACTTTCTGGATGTCTGTGATAATGTTGAACCGCACTTGGTGTG
AATAGTAGCAGCTGTCCTCGATGAGTGGAAAAAGAATGTTTACCATCGAAA
ATAATGCCTGTACCAAAAGTAGTTAATTGCAACATATAGCCCTCATTCCTTG
TTGTCTATCTACCATAGAATCAAGGTAATTACCTTTCTCACATTGAGTAAGA
CCCGCACTAATTCTAAATCGAACAAATCATCAAGTAAAAGT

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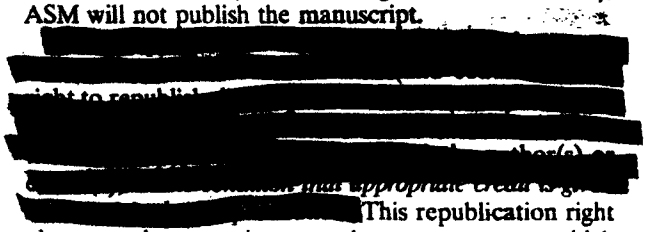

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GenBank: National Center for Biotechnology Information, National Library of Medicine, Bldg. 38A, Rm. 8N-803, Bethesda, MD 20894; telephone, 301-496-2475; fax, 301-480-9241; e-mail, info@ncbi.nlm.nih.gov; URL, http://www.ncbi.nlm.nih.gov.

See p. xii for nucleic acid sequence formatting instructions.

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