Siderophore-independent acquisition of transferrin-bound iron by *Haemophilus influenzae* type b

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The specificity by which *Haemophilus* species acquired iron from transferrin (TF) was investigated. In a plate bioassay *H. influenzae* used iron bound to human, bovine and rabbit TFs but not mouse, rat, dog, horse, guinea-pig, pig or ovo- TFs or human and bovine lactoferrins. In contrast, *H. pleuropneumoniae* used iron only from pig TF whilst *H. parainfluenzae* was unable to utilize iron bound to any of the human or animal TFs tested. The inhibition of growth imposed on *H. influenzae* type b strain Eagan by the addition of the synthetic iron chelator EDDA to the culture medium was reversed by 30% iron-saturated human TF added directly to the medium but not when the TF was contained inside a dialysis bag. Dot-blotting of whole cells revealed that human TF bound to the surface of bacteria cultured in iron-restricted but not in iron-plentiful media. Incubation of whole bacterial cells in the presence of the proteolytic enzyme trypsin also abolished TF-binding activity, suggesting that the TF receptor was a protein. In competition dot blotting experiments, human and bovine but not rabbit, dog, mouse or guinea-pig TFs blocked the binding of a horseradish peroxidase–human TF conjugate. SDS-PAGE and Western blotting of outer membranes revealed the presence of a TF-binding protein of approximately 72 kDa. These results suggest that the acquisition of TF-bound iron by *H. influenzae* type b probably involves a direct interaction with an outer-membrane protein which shows some TF-species specificity.

Introduction

Pathogenic bacteria must not only evade host defences but also successfully compete for nutrients (Brown & Williams, 1985; Williams, 1988). Much evidence has accumulated to suggest that the ability to acquire iron *in vivo* is a critical determinant in pathogenesis (Bullen & Griffiths, 1987; Williams, 1988; Payne, 1988). In experimental animals, parenteral administration of iron enhances the virulence of many bacteria (Bullen & Griffiths, 1987; Payne, 1988). This is because iron, in extracellular body fluids, is normally rendered unavailable to an infecting bacterium by the iron-binding glycoproteins transferrin (TF) (in serum) and lactoferrin (on mucosal surfaces and in polymorphonuclear leucocytes) (Bezkorovainy, 1987). Since these iron-transporting proteins have association constants of around 10^{22} and are only partially saturated with iron (as Fe^{3+}), they maintain the level of free ionic Fe^{3+} far below that required to sustain bacterial growth. Thus the iron-

withholding function of the TFs can be regarded as a non-specific host defence mechanism (Bullen & Griffiths, 1987).

Many Gram-negative pathogens such as *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* overcome this iron restriction by synthesizing high-affinity iron-sequestering systems based on low-molecular-mass iron chelators – siderophores (Bullen & Griffiths, 1987; Williams, 1988; Payne, 1988). These are capable of removing transferrin-bound iron and transporting it into the bacterial cell via specific outer membrane (OM) protein receptors (Bullen & Griffiths, 1987; Williams, 1988; Payne, 1988). Other pathogens, notably *Neisseria gonorrhoeae* and *N. meningitidis*, do not secrete siderophores but acquire iron bound to TF or lactoferrin via a direct interaction between the iron-binding glycoprotein and the bacterial cell surface (Archibald & DeVoe, 1980; West & Sparling, 1985; McKenna *et al.*, 1988).

Haemophilus species colonize the mucosa of the upper respiratory tract of 50–80% of adults and children (Turk, 1984). Of these species *H. influenzae* type b is by far the most important human pathogen and in children, bacteraemia as a result of infection with type b strains

Abbreviations: TF, transferrin; OM, outer membrane; BHI, brain heart infusion; PPIX, protoporphyrin IX; EDDA, ethylenediamine di-*o*-hydroxyphenylacetic acid; HRP, horseradish peroxidase.

may lead to sepsis or localized invasive diseases such as meningitis (Turk, 1984). Additionally this organism may cause epiglottitis, pneumonia and otitis media. Non-typable *H. influenzae* strains are frequently responsible for respiratory tract infections in chronic bronchitis and in cystic fibrosis (Turk, 1984; Murphy & Apicella, 1987). In contrast, other species such as *H. parainfluenzae*, *H. paraphrophilus* and *H. haemolyticus* are only rarely identified as the cause of clinical infections (Albritton, 1982; Black *et al.*, 1988). The *Haemophilus* species described above have no non-human hosts (Turk, 1984) although other species are found in animals, amongst which *H. pleuropneumoniae* and *H. parasuis* are important swine pathogens, causing a spectrum of mainly respiratory diseases (Kilian & Biberstein, 1984; Niven *et al.*, 1989).

The virulence of invasive isolates of *Haemophilus* has been correlated with production of the type b capsular polysaccharide (Turk, 1984). Other virulence factors include lipopolysaccharide (Kaplan *et al.*, 1988), IgA proteases (Farley *et al.*, 1986), and fimbriae (Guerina *et al.*, 1985). However, little information is available concerning the mechanism(s) or role of iron sequestration in the pathogenesis of *Haemophilus* infections.

H. influenzae, unlike *H. parainfluenzae* and *H. pleuropneumoniae*, has an absolute requirement for protoporphyrin IX (Kilian & Biberstein, 1984). *In vivo*, this is probably supplied as haem (ferrityprotoporphyrin IX) and can serve as a source of both iron and porphyrins (White & Granick, 1963; Coulton & Pang, 1983). *H. influenzae* is also capable of using haem bound to serum proteins such as haemopexin and albumin as well as myoglobin, haemoglobin and haemoglobin/haptoglobin complexes (Stull, 1987). However, whilst haem can satisfy all the iron requirements of *H. influenzae* (Pidcock *et al.*, 1988), it is present in only trace amounts in serum. Furthermore, concentrations of haem which satisfy porphyrin requirements do not supply enough iron for growth *in vitro* (Coulton & Pang, 1983). Thus *H. influenzae* may possess additional iron-sequestering mechanisms. Normal human serum contains about 30 µM of 30% iron-saturated TF, and therefore represents a potentially large source of iron (Bezkorovainy, 1987). *H. influenzae* can use human TF as its sole source of iron (Herrington & Sparling, 1985; Morton & Williams, 1989b) but does not appear to produce any siderophores (Morton & Williams, 1989b). The present study was undertaken (a) to determine whether the mechanism of TF iron-acquisition by *H. influenzae* type b requires direct contact between the bacterial cell surface and the iron-binding glycoprotein; (b) to determine the TF-species specificity of *Haemophilus* TF iron-acquisition system(s); and (c) to identify any bacterial surface TF-binding component(s).

Methods

Bacteria and growth conditions. *H. influenzae* type b strain Eagan was kindly provided by Professor R. Moxon, Department of Paediatrics, John Radcliffe Hospital, Oxford, UK. *H. parainfluenzae* P205 was a clinical isolate and has been described previously (Williams & Brown, 1986; Morton & Williams, 1989a). *H. pleuropneumoniae* CVL 882 (serotype 2) was obtained from the Central Veterinary Laboratory, Weybridge, Surrey, UK. *Haemophilus* strains were routinely cultured on chocolate blood agar plates (Lab M). For experiments in liquid media, bacteria were grown with aeration at 37 °C in BHIPPIX medium, consisting of brain heart infusion (BHI) broth supplemented with NAD (2.0 µg ml⁻¹) and protoporphyrin IX (PPIX, 0.5 µg ml⁻¹) (Williams & Brown, 1986). Iron restriction was achieved by the addition of 100 µM-ethylenediamine di-*o*-hydroxyphenylacetic acid (EDDA) (Sigma) to BHIPPIX. Before use, EDDA was freed from contaminating iron as described by Rogers (1973). In some experiments, 120 µM-Fe³⁺ (as ferric nitrate) was added to BHIPPIX + EDDA to overcome the iron restriction imposed by EDDA.

Transferrins. Purified human and animal apoTFs and apolactoferrins (except for the porcine TF) were purchased from Sigma. Porcine TF was prepared from serum by an adaptation of the method of Rogers (1967). Briefly, 200 ml pig serum was diluted to 800 ml with 5 mM-Tris/HCl (pH 8.8), and an equal volume of 0.6% w/v 2-ethoxy-6,9-diaminoacridine lactate (Rivanol) in 5 mM-Tris/HCl (pH 8.8) was slowly added with stirring. The precipitate obtained was allowed to settle and the supernatant decanted and applied to a 20 by 3 cm column of DEAE-Sephadex A-50 (Sigma). TF was eluted with 5 mM-Tris/HCl (pH 8.8) containing 0.5 M-NaCl and extensively dialysed against distilled water. After lyophilization, 200 mg of the partially purified pig TF was diluted in 50 ml distilled water containing 2% (v/v) ampholytes (Bio-Rad; pH range 3–10) and applied to a Rotaphor preparative isoelectric focusing apparatus (Bio-Rad). The focused TF was collected, dialysed against distilled water and freeze-dried. Purity assessed by SDS-PAGE was greater than 95%.

ApoTFs were saturated to 30% by addition of a calculated amount of ferric nitrate in a solution containing a tenfold molar excess of sodium citrate followed by extensive dialysis against 150 mM-NaCl, 10 mM-NaHCO₃ in 10 mM-HEPES (pH 7.4). The percentage iron saturation was confirmed using a diagnostic kit for determination of total iron and iron-binding capacity (Sigma).

Utilization of TF-bound iron. To determine which TFs could supply iron to *Haemophilus*, the plate bioassay described by Herrington & Sparling (1985) was used. Approximately 10⁷ bacteria were spread on BHIPPIX containing 170 µM-EDDA and solidified with 1.5% (w/v) agar. Twenty microlitres of a 1 mg ml⁻¹ solution of 30% iron-saturated human, ovo- or animal TF, or lactoferrin, in 10 mM-Tris/HCl (pH 7.4) was added to wells cut in the agar. After overnight incubation at 37 °C, plates were examined for stimulation of growth around the wells. ApoTF and 30 µM-ferric nitrate were included as negative and positive controls respectively.

Experiments to determine whether TF iron-acquisition by *H. influenzae* type b was via secretion of low-molecular-mass iron chelators or required cell surface contact were done as follows. *H. influenzae* was inoculated into culture flasks containing BHIPPIX and 100 µM-EDDA with or without 30% iron-saturated human TF (200 µg ml⁻¹). To some flasks, a dialysis bag (Medicell; molecular mass cutoff approximately 6000 Da) containing either TF (5 mg in 1 ml) or medium (1 ml) was added. Bacteria were aerated at 37 °C and growth monitored by measuring OD₆₀₅.

TF-binding assays. A solid-phase dot enzyme assay based on that described by Schryvers & Morris (1988) was used to examine the

binding of TF to *Haemophilus*. Bacteria grown to late exponential phase were resuspended in 10 mM-Tris/HCl (pH 7.4) to OD₆₀₅ 1.0. Samples (5 or 10 µl) were filtered onto a nitrocellulose membrane in a microfiltration apparatus (Bio-Rad); the membrane was dried at room temperature for 10 min, blocked with 1% (w/v) skimmed milk and then probed with either a horseradish peroxidase (HRP)-human TF conjugate (Stratech Scientific) or biotin-labelled human TF (50 ng ml⁻¹). Biotinylated human TF was prepared by incubation of TF (1 mg ml⁻¹) with *N*-hydroxysuccinimido-biotin (250 µg dissolved in 16 µl dimethylformamide). After incubation for 2 h at 4 °C, the biotinylation reaction was stopped by the addition of 100 µl glycine (10 mg ml⁻¹); the solution was dialysed and then freeze-dried. Dot-blots were developed with 25 µg 4-chloro-1-naphthol ml⁻¹ and 0.01% (v/v) H₂O₂ in 10 mM-Tris/HCl (pH 7.4).

For competition experiments, a range of concentrations of an unconjugated TF (0–5 µM) was mixed with the HRP-transferrin conjugate (500 ng ml⁻¹; 3.6 nM) prior to application to the nitrocellulose membrane. Assays were repeated at least three times.

In some experiments, whole bacterial cells (resuspended to OD₆₀₅ 1.0) were pretreated for 45 min at 37 °C with the proteolytic enzyme trypsin (1 mg ml⁻¹; Sigma) prior to dot-blotting. Digestions were carried out in 10 mM-Tris/HCl pH 8.0.

Preparation of membranes. Bacterial membranes were prepared by sonication of whole cells resuspended to OD₆₀₅ 1.0 in 50 mM-Tris/HCl (pH 7.4) containing the protease inhibitors phenylmethylsulphonyl fluoride (PMSF) (30 µg ml⁻¹) and benzamide (50 µg ml⁻¹) followed by centrifugation at 5000 r.p.m. for 5 min to remove unbroken cells. Membranes were collected by centrifugation at 40000 *g* for 30 min, resuspended in the above buffer and stored at 4 °C. OM proteins were isolated from crude envelopes using 2% (v/v) Triton X-100 as described by van Alphen *et al.* (1983). For some experiments, OMs were also prepared from cells treated with trypsin as described above.

SDS-PAGE and Western blotting. OM proteins were solubilized for 30 min at 37 °C in sample buffer (3%, w/v. SDS, 16%, v/v, glycerol, 0.001%, w/v, bromophenol blue in 0.08 M-Tris/HCl pH 6.8) without 2-mercaptoethanol. Molecular mass marker proteins were solubilized in the same sample buffer with 2% (v/v) 2-mercaptoethanol and denatured by heating at 100 °C for 5 min. Approximately 10 µg protein per lane was loaded onto polyacrylamide gels prepared as described previously (Williams *et al.*, 1984). After electrophoresis, proteins were fixed and stained with Coomassie blue or transferred electrophoretically to nitrocellulose. Western blots were blocked and developed as described above for whole-cell dot-blotting. After colour development the molecular mass of the TF-receptor was determined by counter-staining the nitrocellulose with India ink (Li *et al.*, 1988).

Results

Utilization of TF-bound iron

The ability of both human (*H. influenzae* and *H. parainfluenzae*) and porcine (*H. pleuropneumoniae*) haemophili to obtain iron bound to TFs and lactoferrins isolated from various mammalian species was examined using a plate bioassay (Table 1). *H. influenzae* type b strain Eagan utilized iron bound to human, rabbit and bovine TFs but not that bound to human or bovine lactoferrin or any of the other TF species tested. *H. pleuropneumoniae* only used iron from pig TF whilst *H. parainfluenzae* was unable acquire iron from any of the

Table 1. Ability of *Haemophilus* spp. to acquire iron from transferrins and lactoferrins

+, Ability, –, inability to acquire iron. *H. parainfluenzae* was unable to acquire iron from any of the transferrins or lactoferrins listed.

Transferrin or lactoferrin (Ltf)	<i>H. influenzae</i> type b	<i>H. pleuropneumoniae</i>
Human	+	–
Human Ltf	–	–
Rat	–	–
Rabbit	+	–
Mouse	–	–
Dog	–	–
Horse	–	–
Bovine	+	–
Bovine Ltf	–	–
Guinea-Pig	–	–
Pig	–	+
Ovo-	–	–

TF species tested (Table 1), even after increasing the iron saturation to 70% (data not shown).

The growth kinetics of *H. influenzae* type b strain Eagan in BHIPPIX broth in the presence and absence of the synthetic iron chelator EDDA are shown in Fig. 1. The inhibition of growth imposed by EDDA was reversed upon addition of 120 µM-ferric nitrate (data not shown) or 200 µM of 30% iron-saturated human TF (Fig. 1). However, when the TF added was contained inside a dialysis bag, growth was not restored (Fig. 1). The dialysis bag alone had no effect on growth in iron-plentiful or iron-restricted medium. In contrast to *H. influenzae*, and in confirmation of the plate bioassay results, human TF did not relieve the growth inhibition of *H. parainfluenzae* in BHIPPIX medium containing EDDA (data not shown). The results of these dialysis bag experiments, together with the previous report (Morton & Williams, 1989b) that *H. influenzae* type b does not produce siderophores, suggest that the acquisition of TF-bound iron by *H. influenzae* does not depend on the secretion of a low-molecular-mass iron chelator but may require direct contact between the bacterial cell surface and human TF.

Bacterial cell surface binding of TF

To determine whether human TF could interact directly with the cell surface of *H. influenzae*, whole cells grown under iron-plentiful and iron-restricted conditions were immobilized on nitrocellulose and incubated with either HRP-TF or biotin-labelled TF followed by streptavidin-peroxidase. HRP-TF was detected bound to *H. influenzae* strain Eagan grown under iron-restricted conditions

Fig. 1. Growth kinetics of *H. influenzae* type b strain Eagan in BHIPPIX. ■, Control, no additions; ●, addition of 100 μ M-EDDA; $\triangle$, addition of 100 μ M-EDDA and 200 μ g 30% iron-saturated human TF ml^{-1} ; ○, addition of 100 μ M-EDDA and 30% iron-saturated human TF (5 mg ml^{-1}) contained inside a dialysis bag.

but not under iron-plentiful conditions (Fig. 2a). Similar results were obtained using bacterial membranes or the biotin detection system (data not shown). *H. parainfluenzae* did not bind human TF (data not shown).

In competition assays, human apoTF blocked the binding of HRP-TF, as did bovine apoTF (Fig. 2b). However, whilst 0.16 μ M human TF effectively blocked the binding of HRP-TF, 1.3 μ M bovine TF was required. No competition between rabbit apoTF (Fig. 2c) or 30% iron-saturated rabbit TF (data not shown) and HRP-TF was observed despite the ability of *H. influenzae* type b strain Eagan to use rabbit TF. TF species which the organism was unable to use as an iron source, including dog, horse, mouse and guinea-pig, also failed to block the binding of HRP-TF (Fig. 2c).

Identification of an *H. influenzae* type b OM TF-binding protein

To determine whether *H. influenzae* produced an OM TF-binding protein, whole cells grown in the presence of EDDA were incubated with the proteolytic enzyme trypsin. The cells were then spotted onto nitrocellulose and incubated with HRP-TF. The results in Fig. 2(a) show that protease treatment abolished the binding of TF to whole cells, indicating the involvement of a bacterial cell surface protein.

To identify this TF-binding protein, OM preparations from bacteria grown under iron-replete and iron-restricted conditions were subjected to SDS-PAGE and electroblotted onto nitrocellulose. The blots were then incubated with either HRP-TF or biotin-labelled

Fig. 2. Dot-enzyme assays showing the binding of an HRP-human TF conjugate to whole cells of *H. influenzae* type b strain Eagan immobilized on nitrocellulose. (a) Binding of HRP-TF to bacteria [10 μ l (dots A1, B1, A3, B3) or 5 μ l (dots A2, B2, A4, B4) suspensions] grown in BHIPPIX with 100 μ M-EDDA (A, 1-4) or in BHIPPIX (B, 1-4), without (A1, A2, B1, B2) or with (A3, A4, B3, B4) pre-incubation in the presence of trypsin. (b, c) Competition binding experiments between various apoTFs and the HRP-human TF conjugate for the *H. influenzae* type b TF receptor. (b) A range of concentrations (0-5.0 μ M; indicated to the left of the photograph) of bovine (C) and human (D) apoTFs were mixed with 3.6 nM-HRP-TF and incubated with the bacterial cells (5 μ l per dot, grown in BHIPPIX with 100 μ M-EDDA). (c) A competition binding experiment between mixtures of 3.6 nM-HRP-TF and 5.0 μ M-unconjugated rabbit (R), dog (Do), horse (H), mouse (M) and guinea-pig (GP) TFs.

TF followed by streptavidin-peroxidase. Using the biotinylated detection system, the presence of an OM TF-binding protein of approximately 72 kDa was detected in OM preparations from bacteria grown under iron-restricted conditions (Fig. 3, land D). Pre-treatment of whole cells with trypsin prior to the preparation and Western blotting of OM preparations also

Fig. 3. SDS-PAGE and Western blot showing the binding of biotin-labelled human TF to a 72 kDa *H. influenzae* type b OM protein. OM proteins prepared from bacteria grown in BHIPPIX (lanes A and C) or BHIPPIX with 100 μ M-EDDA (lanes B and D) were electrophoresed on SDS-polyacrylamide gels (12.5%, w/v, acrylamide) and stained with Coomassie blue (lanes A and B) or transferred to nitrocellulose and probed with biotin-labelled human TF followed by streptavidin-peroxidase (lanes C and D).

abolished the TF-binding activity (data not shown). In the absence of biotin-labelled TF, streptavidin-peroxidase did not bind to the putative TF receptor (data not shown). HRP-TF, however, did not react on the Western blots. This may have been due either to the greater sensitivity of the biotin-streptavidin system or to interference of the HRP enzyme label of HRP-TF in the reaction with the partially renatured TF receptor, a problem which is not apparent in dot-blotting experiments.

This 72 kDa TF-binding protein also proved difficult to visualize in Coomassie-blue-stained SDS-PAGE gels. No obvious differences were apparent between the OM proteins of cells grown under iron-plentiful or iron-restricted conditions (Fig. 3, lanes A and B). This suggests that the 72 kDa OM protein is a minor OM protein which may be co-migrating with an OM protein of the same molecular mass present in cells grown under both conditions. The more sensitive silver staining technique also failed to reveal any obvious differences (data not shown).

Discussion

The mechanism by which *H. influenzae* type b utilizes TF-bound iron appears to differ from the siderophore-based high-affinity iron-sequestering systems of the *Enterobacteriaceae*. In response to iron stress, *H. influenzae* type b does not synthesize siderophores (Morton &

Williams, 1989b) or major iron-repressible OM proteins (Williams & Brown, 1986; Morton & Williams, 1989b), nor does it use exogenously supplied siderophores such as enterochelin or aerobactin (unpublished observations), or desferrioxamine (Williams & Brown, 1986). Possible alternative mechanisms could include (a) proteolytic degradation of transferrin to release the iron or (b) a direct interaction between the iron-binding glycoprotein and the bacterial cell surface. Growth of *H. influenzae* type b in media containing human TF does not, however, lead to the proteolytic degradation of the iron-binding glycoprotein (unpublished observation). Thus, the failure to detect siderophores (Morton & Williams, 1989b), and the inability of *H. influenzae* type b to obtain iron from TF when the TF was separated from the bacterium by a dialysis membrane, suggested that the organism may require direct surface contact with the iron-binding glycoprotein. Such a siderophore-independent mechanism has been reported to operate in *N. meningitidis* (Tsai *et al.*, 1988) and *N. gonorrhoeae* (McKenna *et al.*, 1988).

Further evidence that acquisition of TF-bound iron by *H. influenzae* requires direct cell surface contact was obtained using a solid-phase dot enzyme assay based on the interaction between HRP-TF or biotin-labelled TF and whole cells immobilized on nitrocellulose. Bacteria cultured under iron-restricted but not under iron-plentiful conditions bound labelled TF. In addition, since apoTF blocked the binding of HRP-TF, the receptor does not appear to show a preference for the ferrated form of TF. Similar results have been reported for the *N. meningitidis* TF receptor (Tsai *et al.*, 1988) although others have reported that iron saturation influences the interaction between TF and its receptor in both *N. meningitidis* (Schryvers & Morris, 1988) and *H. influenzae* (Schryvers, 1988). These discrepancies may prove to be strain dependent or may have arisen because of differences in the methodologies used. However, it has been suggested that since TF is only about 30% saturated under physiological conditions, a low-affinity receptor permitting high turnover of TF at the cell surface may confer a greater advantage by enabling the bacterium to sort through the available TF for ferrated molecules (Tsai *et al.*, 1988). Work is currently under way to determine the affinity and copy number of the *H. influenzae* TF receptor and whether TF, once bound, is internalized. Interestingly, pathogenic *Neisseria* species, unlike eukaryotic cells, do not appear to internalize TF (Archibald & DeVoe, 1979; McKenna *et al.*, 1988).

Since *H. influenzae* type b has no natural non-human hosts (Turk, 1984), we also examined its TF-species specificity. Of the ten TFs and two lactoferrins tested, only human, rabbit and bovine TFs supplied iron to strain Eagan. Both bovine and human TFs blocked the

binding of HRP-TF in the whole-cell solid-phase assay although human TF competed more efficiently. Other *H. influenzae* type b strains have been reported not to bind bovine TF, although their use of iron bound to this TF was not examined (Schryvers, 1988). We were unable to demonstrate that rabbit TF could compete with HRP-TF, perhaps indicating the existence of an alternative receptor or mechanism of TF iron acquisition.

The inability of strain Eagan to use rat TF was unexpected, since the best model of *Haemophilus meningitis* in humans is the infant rat (Moxon, 1985). In this study, however, we used a commercial source of adult rat TF, and since not all rat strains are susceptible to *Haemophilus*, work is under way to isolate and examine the utilization of TF from susceptible infant rats.

Thus, although it is exclusively a human pathogen, *H. influenzae* type b is not restricted to using human TF. Whether the organism recognizes part of the amino acid sequence, an oligosaccharide substituent or both has not been elucidated. However, since several TFs have closely related amino acid sequences but differ in the position and type of oligosaccharide substituent (Bezkorovainy, 1987), it is tempting to speculate that the carbohydrate moieties play an important role.

Amongst less pathogenic human haemophili such as *H. parainfluenzae*, *H. paraphrophilus* and *H. haemolyticus*, only the latter species has been reported to use iron bound to human TF (Morton & Williams, 1989b). However, whilst pathogenic non-human haemophili such as the swine pathogens *H. pleuropneumoniae* and *H. parasuis* were unable to use iron from human TF, human lactoferrin or ovotransferrin they could use porcine TFs (Morton & Williams, 1989b; Niven *et al.*, 1989). In this study, we examined a wider range of TFs and found that only porcine TF supplied iron to *H. pleuropneumoniae*. Since this organism does not appear to produce siderophores (Morton & Williams, 1989b), further work is required to determine whether it expresses a TF receptor with a more restricted host TF requirement than *H. influenzae*.

Pre-incubation of *H. influenzae* type b with trypsin abolished the binding of labelled human TF to immobilized bacteria, suggesting that the TF receptor is a protein. In Western blots, biotinylated TF reacted with a single protein in OM preparations from strain Eagan grown under iron-restricted conditions. The apparent molecular mass of this putative OM TF receptor was about 72 kDa. OM proteins in the 65–85 kDa range which bind HRP-TF in Western blots have been observed in *N. meningitidis* (Tsai *et al.*, 1988). However, an *N. meningitidis* mutant lacking a 65 kDa HRP-TF binding protein in Western blots nevertheless exhibited normal TF receptor activity (Tsai *et al.*, 1988). Thus, the precise contribution

of the 72 kDa protein to TF receptor function and iron acquisition in *H. influenzae* remains to be established.

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