Utilization of enterobactin and other exogenous iron sources by *Haemophilus influenzae*, *H. parainfluenzae* and *H. paraphrophilus*

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The ability of *Haemophilus influenzae*, *H. parainfluenzae* and *H. paraphrophilus* to utilize iron complexes, iron-proteins and exogenous microbial siderophores was evaluated. In a plate bioassay, all three species used not only ferric nitrate but also the iron chelates ferric citrate, ferric nitrilotriacetate and ferric 2,3-dihydroxybenzoate. Each *Haemophilus* species examined also used haemin, haemoglobin and haem-albumin as iron sources although only *H. influenzae* could acquire iron from transferrin or from haemoglobin complexed with haptoglobin. None of the haemophili obtained iron from ferritin or lactoferrin or from the microbial siderophores aerobactin or desferrioxamine B. However, the phenolate siderophore enterobactin supplied iron to both *H. parainfluenzae* and *H. paraphrophilus*, and DNA isolated from both organisms hybridized with a DNA probe prepared from the *Escherichia coli* ferric enterobactin receptor gene *fepA*. In addition, a monospecific polyclonal antiserum raised against the *E. coli* 81 kDa ferric enterobactin receptor (FepA) recognized an iron-repressible outer membrane protein (OMP) in *H. parainfluenzae* of between 80 and 82 kDa (depending on the strain). This anti-FepA serum did not cross-react with any of the OMPs of *H. paraphrophilus* or *H. influenzae*. The OMPs of each *Haemophilus* species were also probed with antisera raised against the 74 kDa Cir or 74 kDa IutA (aerobactin receptor) proteins of *E. coli*. Apart from one *H. parainfluenzae* strain (NCTC 10665), in which an OMP of about 80 kDa cross-reacted with the anti-IutA sera, no cross-reactivity was observed between Cir, IutA and the OMPs of *H. influenzae*, *H. parainfluenzae* or *H. paraphrophilus*. Thus, *H. parainfluenzae* and *H. paraphrophilus* possess a functional enterobactin iron-uptake system, and both siderophore-dependent and siderophore-independent high-affinity iron-sequestering systems are now known to be expressed in the genus *Haemophilus*.

Introduction

Haemophilus species, including *H. influenzae*, *H. parainfluenzae* and *H. paraphrophilus*, are common commensals of the human upper respiratory tract (Kilian & Biberstein, 1984). *H. influenzae*, especially type b, is by far the most important human pathogen responsible for meningitis, epiglottitis, pneumonia and otitis media (Turk, 1984). *H. parainfluenzae* and *H. paraphrophilus* are relatively rare causes of clinical infections (Albritton, 1982) although the increased reporting of *H. parainfluen-*

zae paediatric infections (Black *et al.*, 1988; Quentin *et al.*, 1989) requires that the pathogenic potential of this organism should not be overlooked.

The virulence of invasive isolates of *Haemophilus* has been correlated with production of the type b capsular polysaccharide (Turk, 1984; Moxon, 1985), lipopolysaccharide (Kaplan *et al.*, 1988), IgA proteases (Farley *et al.*, 1986) and fimbriae (Guerina *et al.*, 1985). However, since bacteria need to multiply *in vivo* to establish an infection, the ability to acquire essential nutrients can also be regarded as a potential virulence determinant (Bullen & Griffiths, 1987; Williams, 1988). Haemophili are characterized by their requirements for either or both porphyrins [as protoporphyrin IX (PPIX) or ferriprotoporphyrin (haem)] and pyridine nucleotides (e.g. NAD) (Kilian & Biberstein, 1984). *H. influenzae*, unlike *H. parainfluenzae* and *H. paraphrophilus*, has an absolute

Abbreviations: OMP, outer-membrane protein; IROMP, iron-repressible outer-membrane protein; PPIX, protoporphyrin IX; EDDA, ethylenediamine-di-*o*-hydroxyphenylacetic acid; BHIPN, brain heart infusion broth containing PPIX and NAD; DHBA, 2,3-dihydroxybenzoic acid.

requirement for PPIX, which it can use to synthesize haem via the enzyme ferrochelatase (White & Granick, 1963). However, since PPIX is an intracellular intermediate in mammalian haem synthesis (Israels *et al.*, 1975), haem rather than PPIX is more likely to supply *H. influenzae* with the essential porphyrin ring in extracellular body fluids. Haem in excess of porphyrin requirements can also function as an iron supply for *H. influenzae* (Coulton & Pang, 1983). However, free haem compounds are not readily available in plasma since any haemoglobin liberated by haemolysis is rapidly bound by haptoglobin whilst any free haem liberated by the dissociation of haemoglobin is avidly bound by haemopexin and/or less tightly by serum albumin (Muller-Eberhard, 1970). Similarly, although there is plenty of iron in extracellular body fluids, it too, is not readily available to an infecting bacterium since it is tightly complexed to iron-binding glycoproteins known as transferrins (Bezborovainy, 1987).

Little is known of the mechanisms by which bacteria acquire iron as haem. However, under conditions of iron stress imposed by the presence of transferrins, many Gram-negative bacteria derepress high-affinity iron-uptake systems capable of sequestering the glycoprotein-bound iron. These systems consist of low-molecular-mass iron-chelators (siderophores) and their outer-membrane protein (OMP) receptors (Griffiths, 1987; Williams, 1988). In response to iron deprivation *E. coli* and other members of the *Enterobacteriaceae* synthesize the phenolate siderophore enterobactin (enterochelin), a cyclic triester of 2,3-dihydroxybenzoylserine which is capable of solubilizing transferrin-bound iron and transporting it back into the bacterial cell via an 81 kDa outer-membrane receptor protein designated FepA (Griffiths, 1987; Payne, 1988; Crosa, 1989). Some *E. coli* strains also synthesize an additional 74 kDa iron-regulated protein called IutA, which is the receptor for ferric aerobactin, a citrate-hydroxamate siderophore, the production of which has been reported to enhance the virulence of both *E. coli* and *Klebsiella pneumoniae* (Payne, 1988).

In contrast, *Neisseria meningitidis* and *N. gonorrhoeae* express a siderophore-independent iron-uptake mechanism based on the direct interaction between transferrin and a cell surface receptor (Tsai *et al.*, 1988; McKenna *et al.*, 1988). We have recently reported that a similar system operates in *H. influenzae* (Morton & Williams, 1989a, 1990). However, other human haemophili, such as *H. parainfluenzae* and *H. paraphrophilus*, appear unable to acquire transferrin-bound iron despite the apparent expression of a siderophore-mediated iron uptake system (Morton & Williams, 1989b). For example, in response to iron stress, *H. parainfluenzae* produces iron chelators and up to four iron-repressible OMPs in the 70–90 kDa range (Williams & Brown, 1986; Morton

& Williams, 1989b). These iron-repressible OMPs (IROMPs) could function as receptors for siderophores produced by other micro-organisms. The present study was undertaken to determine: (a) whether siderophores such as enterobactin and aerobactin could be utilized by *H. influenzae*, *H. parainfluenzae* or *H. paraphrophilus*; (b) the relationship, if any, between the IROMPs of *E. coli* and those of *Haemophilus*; and (c) which mammalian haem and non-haem iron sources could be utilized by different *Haemophilus* species.

Methods

Bacterial strains and growth conditions. *H. influenzae* type b strain Eagan, *H. parainfluenzae* strains P205, HK115, HK116, HK255, NCTC 7857 and NCTC 10665, and *H. paraphrophilus* HK319 have all been described previously (Morton & Williams, 1989a, b). *H. paraphrophilus* strains HK319 and HK415 (as well as *H. parainfluenzae* strains HK115, and HK116 and HK255) were kindly provided by Professor M. Kilian (Royal Dental College, Aarhus, Denmark). The *E. coli* strains K12 (pColV-K30) and H247 (O18:K1:H7, ColV⁺; Chart & Griffiths, 1985), both of which possess aerobactin and enterobactin iron-uptake systems, were used as controls in some experiments. All strains were routinely subcultured at 37 °C on chocolate blood agar. For experiments in liquid media, bacteria were grown with aeration in BHIPN, which consists of brain heart infusion broth supplemented with NAD (2 µg ml⁻¹) and PPIX (0.5 µg ml⁻¹). Iron restriction was achieved by the addition of 100 µM-ethylenediamine-di-o-hydroxyphenylacetic acid (EDDA, Sigma). Before use, EDDA was freed from contaminating iron as described by Rogers (1973).

Utilization of iron compounds. To determine which iron compounds could supply iron to *Haemophilus*, the plate bioassay described previously (Morton & Williams, 1990) was used. Approximately 10⁷ bacteria were spread on BHIPN containing 170 µM-EDDA and solidified with 1.5% (w/v) agar. Portions (20 µl) of the test iron compounds (at concentrations of 20 mM for iron salts or 1 mg ml⁻¹ for protein-bound iron) were added to wells cut in the agar. Plates were examined for stimulation of growth around the wells after overnight incubation at 37 °C.

The following compounds were evaluated for their ability to supply iron to *Haemophilus*: ferric nitrate, ferric citrate, ferric nitrioltriacetate, enterobactin, 2,3-dihydroxybenzoic acid (DHBA), aerobactin, desferrioxamine B, human transferrin (saturated to 30% and 90% with iron), human lactoferrin (saturated to 30% and 90% with iron), haemin, haemoglobin, haptoglobin-haemoglobin, haem-albumin, and ferritin. All iron compounds were purchased from Sigma unless otherwise stated. Enterobactin and aerobactin were isolated from *E. coli* K12 (pColV-K30) as described by O'Brien & Gibson (1970) and Williams *et al.* (1989) respectively. Desferrioxamine was purchased from Ciba-Geigy. Human transferrin and lactoferrin were saturated to 30% or 90% with iron as described previously (Morton & Williams, 1990). The preparation of haem-albumin (methaemalbumin) and haptoglobin-haemoglobin complexes was as described by Stull (1987). Where required, apotransferrin, apolactoferrin, albumin and haptoglobin were evaluated as negative controls.

Preparation of fepA DNA probe. Plasmid pITS449 (kindly provided by Dr S. K. Armstrong, Department of Microbiology, University of Missouri-Columbia, USA) contains a 2.5 kb *Spl*-*Stu*I fragment from plasmid pITS1 (which contains the *E. coli* enterobactin system genes *entD*, *fepA* and *fes*; Fleming *et al.*, 1985; Armstrong *et al.*, 1989) cloned

into the *Sma*I site of pUC18 (Murphy & Klebba, 1989). The probe comprised the 2.5 kb *Sma*I fragment of pITS449 and consisted almost entirely of the *fepA* gene. Plasmid DNA was isolated and digested with *Sma*I as described previously (Carter *et al.*, 1987a). The 2.5 kb fragment was recovered by electroelution from a horizontal 0.7% agarose slab gel and labelled with biotin-14-dATP using a BioNick Translation Kit (Gibco/BRL) and the conditions recommended by the manufacturer.

Detection of enterobactin receptor genes. Each *Haemophilus* species was screened for the presence of a ferric enterobactin receptor gene using the dot-blot hybridization technique. For dot-blots, total cellular DNA was isolated from *Haemophilus* using the method of Hoiseth *et al.* (1985). DNA prepared from *E. coli* was included as a positive control. DNA samples (100 µl in TE buffer: 10 mM-Tris, 1 mM-EDTA) were filtered under negative pressure through a 96-well Bio-Dot Microfiltration apparatus (Bio-Rad laboratories) onto a hydrated nitrocellulose membrane. Filters were air-dried and then baked at 80 °C for 2 h to bind the DNA to the membrane. Prior to hybridization, filters were re-hydrated and treated with proteinase K as described by Carter *et al.* (1987b). Pre-hybridization, and hybridization with the biotin-labelled *fepA* probe, were also as described previously (Carter *et al.*, 1987b). Detection of a positive hybridization result was by means of a BlueGENE Kit (Gibco/BRL) using the conditions and protocols recommended by the manufacturer. These include high-stringency wash conditions that require nucleotide sequence homology in excess of 75% to generate a positive result (K. J. Towner, unpublished results).

Preparation of outer membranes. Outer membranes were prepared from *Haemophilus* and *E. coli* as described by Morton & Williams (1989a, b) using 2% (w/v) sodium *N*-lauroyl sarcosine (Sarkosyl) to remove cytoplasmic membrane material from envelopes prepared by sonication of whole cells.

SDS-PAGE and immunoblotting. OMPS were separated on 10% (w/v) SDS-polyacrylamide gels (approximately 10 µ protein per lane). Following electrophoresis, gels were either fixed and stained with Coomassie Brilliant Blue R250 or used for immunoblotting. OMPs were transferred to nitrocellulose membranes and reacted with a polyclonal antiserum, raised in rabbits as described in the accompanying references, against the following *E. coli* IROMPs: (a) the 81 kDa ferric enterobactin receptor FepA (Chart & Griffiths, 1985); (b) the 74 kDa Cir protein (Griffiths *et al.*, 1985); and (c) the 74 kDa aerobactin receptor IutA (Williams *et al.*, 1989). Rabbit antibody was detected with protein A-horseradish peroxidase (Sigma). Antigenic sites were visualized with a 25 µg ml⁻¹ solution of 1-chloro-4-naphthol containing 0.01% (v/v) H₂O₂. The molecular masses and positions of cross-reacting OMPs were then confirmed by counter-staining the immunoblots with 0.5% (w/v) Ponceau S in 1% (v/v) acetic acid.

Results

Utilization of iron salts and microbial siderophores

The ability of *Haemophilus* to overcome the iron-restriction imposed by 170 µM-EDDA by utilizing several different iron complexes and microbial iron chelators was examined. The results (summarized in Table 1) show that all three *Haemophilus* species examined could use iron not only when supplied as ferric nitrate but also when supplied as ferric citrate and ferric nitrilotriacetate, two compounds which efficiently chelate iron. The phenolate siderophore enterobactin also stimulated the growth of *H. parainfluenzae* P205 and *H. paraphrophilus* HK319 but not that of *H. influenzae*. Similar results were obtained with the five other strains of *H. parainfluenzae* used in this study (data not shown). Interestingly, DHBA, a precursor of enterobactin and a weak iron chelator, was also able to reverse the growth inhibition not only of *H. parainfluenzae* and *H. paraphrophilus* but also of *H. influenzae*, but only when supplied as the iron chelate. None of the *Haemophilus* strains tested were capable of utilizing the hydroxamate microbial siderophores aerobactin or desferrioxamine B.

Hybridization of the *fepA* DNA probe with *Haemophilus* DNA

A DNA probe prepared from the *E. coli* ferric enterobactin receptor gene *fepA* was used in a dot-blot assay to determine whether there was DNA homology between the *E. coli* receptor and the putative receptors in *H. parainfluenzae* and *H. paraphrophilus*. The probe hybridized with DNA prepared from six *H. parainfluenzae* and two *H. paraphrophilus* strains, but not with DNA from *H. influenzae* type b strain Eagan (Table 2). In addition, DNA isolated from the non-typable *H. influenzae* strain 11PS did not hybridize with the DNA probe (data not shown).

Table 1. Utilization of iron compounds and exogenous microbial siderophores by *Haemophilus* spp.

Strain	Utilization of*:						
	FeN	FeC	FeNTA	FeDHB	Ent	Aer	Des
<i>H. influenzae</i> Eagan	+	+	+	+	—	—	—
<i>H. parainfluenzae</i> P205	+	+	+	+	+	—	—
<i>H. paraphrophilus</i> HK319	+	+	+	+	+	—	—

*Key: +, —, able or unable to utilize as an iron source; FeN, ferric nitrate; FeC, ferric citrate; FeNTA, ferric nitrilotriacetate; FeDHB, ferric dihydroxybenzoate; Ent, enterochelin; Aer, aerobactin; Des, desferrioxamine.

Fig. 1. SDS-PAGE of the OMPs of *E. coli* H247 (lane A); *H. influenzae* Eagan (lanes B and C); *H. paraphrophilus*, strains HK319 (lanes D and E) and HK415 (lane F); and *H. parainfluenzae* strains P205 (lanes G and H); NCTC 10665 (lane I), HK255 (lane J), 7857 (lane K), HK115 (lane L) and HK116 (lane M). Outer membranes were prepared from bacteria grown in iron-sufficient conditions (lanes B, D and G) or under iron-restricted conditions (lanes A, C, E, F, H–M). Lane N, molecular mass markers. Arrowheads indicate the positions of the IROMPs in *H. parainfluenzae* which cross-react with the *E. coli* ferric enterobactin receptor.

Table 2. Relationships between the IROMPs of *E. coli* and *Haemophilus* spp.

Key: +, Positive reaction; –, negative reaction; *fepA*, hybridization with the *fepA* ferric enterobactin receptor DNA probe; FepA, IutA and Cir, cross-reactivity in immunoblots of polyclonal antisera raised against the 81 kDa ferric enterobactin receptor, the 74 kDa ferric aerobactin receptor and the 74 kDa Cir protein of *E. coli*, respectively, and the IROMPs of *Haemophilus*.

Species (and no. of strains tested)	<i>fepA</i>	FepA	IutA	Cir
<i>H. influenzae</i> (1)	–	–	–	–
<i>H. parainfluenzae</i> (6)	+	+	–*	–
<i>H. paraphrophilus</i> (2)	+	–	–	–

*An IROMP of about 80 kDa in *H. parainfluenzae* NCTC 10665 reacted with the antiserum raised against IutA.

Comparison of the IROMPs of *E. coli* and *Haemophilus*

Fig. 1 shows an SDS-PAGE gel of the OMPs of *H. influenzae*, *H. parainfluenzae* and *H. paraphrophilus* grown under iron-restricted conditions and compared with those of *E. coli*. The OMPs of *H. influenzae* (Eagan), *H. parainfluenzae* P205 and *H. paraphrophilus* HK319 grown under iron-sufficient conditions (Fig. 1, lanes B, D and G) are included for comparison. Whilst *E. coli* produces two strongly induced IROMPS, of 81 kDa (FepA) and 74 kDa (Cir and IutA), the OMP profile of *H. influenzae* showed little change in response to iron deprivation. However, both *H. parainfluenzae* and *H. paraphrophilus*

expressed up to four IROMPs in the 70–90 kDa range, which were repressed upon addition of excess iron to the medium.

Immunochemical cross-reactivity of the *E. coli* and *Haemophilus* IROMPs

Monospecific polyclonal antisera raised against the purified *E. coli* FepA, Cir and IutA IROMPs were used to probe immunoblots of the OMPs of *Haemophilus* separated on SDS-PAGE. The results obtained are shown in Figs 2 and 3 and summarized in Table 2. In each of the six *H. parainfluenzae* strains examined, an IROMP was recognized by the anti-FepA sera (Fig. 2, lanes G–L). This IROMP showed some small variations in molecular mass in that it appeared to be approximately 81 kDa in *H. parainfluenzae* strains P205, HK115 and HK116, 82 kDa in strains NCTC 10665 and HK255 and 80 kDa in strain NCTC 7857. No reaction was observed with the OMPs of *H. parainfluenzae* P205 cultured under iron-sufficient conditions (Fig. 2, lane F). In addition, no cross-reactivity between the anti-FepA serum and the IROMPs of *H. paraphrophilus* (Fig. 2, lanes C, D and E) or with *H. influenzae* (Fig. 2, lane B) cultured under iron-restricted conditions was observed.

Immunoblots of the OMPs of *H. influenzae*, *H. paraphrophilus* and *H. parainfluenzae* were also probed with antisera raised against the *E. coli* IutA and Cir proteins. The results (Table 2, Fig. 3) show that apart from *H. parainfluenzae* NCTC 10665, no antigenic cross-reactivity was observed between the anti-Cir or anti-

Fig. 2. Immunoblot of the OMPs of *E. coli* H247 and *Haemophilus* spp. showing the cross-reactivity of a polyclonal antiserum raised against the *E. coli* 81 kDa ferric enterobactin receptor protein. Lane A, *E. coli* H247; lane B, *H. influenzae* strain Eagan; lanes C and D, *H. paraphrophilus* HK319; lane E, *H. paraphrophilus* HK415; lanes F and G, *H. parainfluenzae* P205; lanes H, I, J, K, L, *H. parainfluenzae* strains HK255, NCTC 10665, NCTC 7857, HK115, HK116 respectively. Outer membranes were prepared from bacteria grown under iron-restricted conditions apart from those shown in lanes C and F, which were from iron-sufficient conditions.

Fig. 3. Immunoblot of the OMPs of *E. coli* K12 (pColV-K30) (lane A) and *H. parainfluenzae* strains P205 (lane B), HK255 (lane C), NCTC 10665 (lane D), NCTC 7857 (lane E), HK115 (lane F) and HK116 (lane G) showing the reaction of a polyclonal antiserum raised against the pColV-K30 74 kDa ferric aerobactin receptor. All strains were grown under iron-restricted conditions.

IutA serum and any of the *Haemophilus* strains examined. An IROMP of about 80 kDa in *H. parainfluenzae* NCTC 10665 showed some cross-reactivity with the anti-IutA serum (Fig. 3, lane D).

Utilization of mammalian iron-containing compounds

Haemin and a variety of haem and non-haem iron-containing proteins found in vertebrate hosts were tested for their ability to relieve the iron-restriction imposed on haemophili by EDDA. Amongst these, haemin, haemoglobin and haem-albumin stimulated the growth of all three species (Table 3). *H. influenzae*, unlike *H. parainfluenzae* or *H. paraphrophilus*, was able to utilize haemoglobin bound to haptoglobin as an iron source. As in previous reports (Herrington & Sparling, 1985; Morton & Williams, 1989a, 1990), human transferrin, but not lactoferrin, was used as an iron source by *H. influenzae*, but not by *H. parainfluenzae* or *H. paraphrophilus*. None of the *Haemophilus* strains tested were capable of utilizing ferritin as an iron source.

Discussion

The growth of haemophili on mammalian mucosal surfaces or in extracellular body fluids will depend, at least in part, upon the acquisition of certain essential nutrients including iron, porphyrins and pyridine nucleotides. These may be obtained either from the host or from other commensal micro-organisms. Potential mammalian iron and haem sources include haem proteins (e.g. haemoglobin) and additional iron supplies may be provided by iron-transporting proteins such as transferrin and ferritin. Furthermore, in mixed commensal

Table 3. Utilization of mammalian haem and non-haem iron proteins by *Haemophilus* spp.

Strain	Utilization of*:							
	HTf30	HTf90	HLf	Ha	Hb	Hb-Hp	HMA	Ft
<i>H. influenzae</i> Eagan	+	+	-	+	+	+	+	-
<i>H. parainfluenzae</i> P205	-	-	-	+	+	-	+	-
<i>H. paraphrophilus</i> HK319	-	-	-	+	+	-	+	-

*Key: +, -, able or unable to utilize as an iron source; HTf30, HTf90, human transferrin saturated to 30% and 90% with iron, respectively; HLf, human lactoferrin; Ha, haemin; Hb, haemoglobin; Hb-Hp, haemoglobin-haptoglobin complex; HMA, haem-albumin; Ft, ferritin.

microbial populations, siderophores produced by one micro-organism may be exploited as iron sources by another. In the present study, the ability of three *Haemophilus* species to utilize a variety of iron and haem compounds as iron sources was evaluated. Only *H. influenzae* was found to be capable of sequestering both transferrin-bound iron and iron from haemoglobin complexed with haptoglobin. In a haem-depleted, iron-sufficient medium, *H. influenzae* has also been reported to obtain haem from the plasma haem-binding protein haemopexin as well as from the primarily intracellular proteins cytochrome *c* and myoglobin (Stull, 1987). Preliminary experiments have shown that myoglobin, for example, can stimulate the growth of *H. influenzae* in media containing an inhibitory concentration of EDDA. Pidcock *et al.* (1988) have also reported that haem-haemopexin, haemoglobin-haptoglobin and haem-albumin complexes could serve as iron sources for *H. influenzae* cultured under iron-restricted conditions. Although the mechanism(s) by which haemophili acquire haem iron have not been elucidated, *H. influenzae* type b sequesters transferrin-bound iron via a siderophore-independent mechanism involving a direct interaction between the iron-binding glycoprotein and an OMP receptor (Morton & Williams, 1989a, 1990). Further work is required to determine whether the *H. influenzae* outer membrane also contains additional receptors for haem-proteins. The presence of haem-repressible OMPs of as yet unidentified function has previously been noted in both type b (Coulton & Pang, 1983; Williams & Brown, 1986; Stull, 1987) and non-typable *H. influenzae* strains (MacIver *et al.*, 1990). The contribution of such systems to the virulence of *H. influenzae* has not yet been established. However, many patients with invasive *H. influenzae* type b infections develop a haemolytic anaemia (Shurin *et al.*, 1986) resulting in the release of intracellular haemoglobin, which in serum, will rapidly be bound by haptoglobin (Bezkorovainy, 1987). This haemoglobin-binding protein has previously been reported to reduce the virulence

of invasive *E. coli* strains by rendering haem iron unavailable (Eaton *et al.*, 1982). In this respect, it is perhaps worth noting that although each of the three *Haemophilus* species examined could acquire iron from free haemoglobin, only the major pathogen, i.e. *H. influenzae* type b, was capable of sequestering haemoglobin bound to haptoglobin.

Despite their inability to sequester transferrin-bound iron in the bioassay employed, both *H. parainfluenzae* and *H. paraphrophilus* produced several IROMPs in the 70–90 kDa range (Williams & Brown, 1986; Morton & Williams, 1989a, b) in response to iron stress. These IROMPs could act as receptors for endogenous or exogenous siderophores. Production of iron chelators by these organisms, but not by *H. influenzae*, has previously been reported (Morton & Williams, 1989a). Following growth of *H. parainfluenzae* in an iron-deficient chemically defined medium, phenolate but not hydroxamate iron-chelators could be detected in the spent supernatant, suggesting that the organism may produce a phenolate-type siderophore (unpublished observation). This iron chelator does not appear to be enterobactin, since the ability to produce enterobactin would enable *H. parainfluenzae* and *H. paraphrophilus* to utilize transferrin-bound iron (Ford *et al.*, 1988) as well as to overcome growth inhibition imposed by EDDA (at the concentration used in this study, i.e. 170 µM). Further work is under way to elucidate the structure and function of this putative siderophore.

Enteric bacteria are known to express receptors not only for their own siderophores, but also for those of other micro-organisms (Griffiths, 1987). For example, *E. coli* is capable of using fungal chelators such as ferri-chrome and coprogen (Griffiths, 1987), whilst *N. gonorrhoeae* can use aerobactin (West & Sparling, 1985). The ability of haemophili to use exogenous siderophores was therefore examined. *H. parainfluenzae* and *H. paraphrophilus*, but not *H. influenzae*, were shown in a bioassay to use enterobactin, but not aerobactin or desferrioxamine B. Similar results have previously been

reported for *H. influenzae*, by Pidcock *et al.* (1988) for enterobactin and by Williams & Brown (1986) for desferrioxamine.

The uptake of enterobactin by *H. parainfluenzae* and *H. paraphrophilus* could be due either to the possession of an uptake system for phenolates in general or to the presence of a specific enterobactin receptor. DNA isolated from *H. parainfluenzae* and *H. paraphrophilus*, hybridized with a DNA probe for the *E. coli* ferric enterobactin receptor gene, indicating the existence of a closely related receptor in these haemophili. Further evidence to support this conclusion was obtained from immunoblotting experiments using a monospecific antiserum raised against the *E. coli* enterobactin receptor. This antiserum reacted with an IROMP in *H. parainfluenzae* of 80–82 kDa, depending on the strain. In *H. parainfluenzae* this IROMP is therefore probably the ferric enterobactin receptor, as previous studies have demonstrated a high degree of antigenic homology of this receptor not only in *E. coli* but also in *Salmonella typhimurium* and *K. pneumoniae* (Chart & Griffiths, 1985; Williams *et al.*, 1987). However, there appears to be some divergence in the ferric enterobactin receptor of *H. paraphrophilus* since the anti-FepA serum did not react in immunoblots with any of its IROMPS. The transport and intracellular release of iron from ferric enterobactin in *E. coli* is also known to involve at least six other genes (Crosa, 1989). Further work is required to determine whether the other genes involved in ferric enterobactin uptake in *H. parainfluenzae* and *H. paraphrophilus* are homologous with those of *E. coli*.

In immunoblotting experiments, no antigenic homology between the *H. parainfluenzae* and *H. paraphrophilus*, IROMPs and the 74 kDa Cir IROMP was observed and only one of six *H. parainfluenzae* strains examined showed any cross-reactivity with the anti-74 kDa IutA IROMP. The lack of any antigenic homology with IutA was perhaps not unexpected since neither organism was able to use ferric aerobactin. However in *E. coli* both Cir and a 83 kDa IROMP (Fiu) have recently been reported to play a role in the uptake of 2,3-dihydroxybenzoylserine (DHBS) and 2,3-dihydroxybenzoic acid (DHBA), two breakdown products of enterobactin which possess weak iron-chelating properties (Hantke, 1990). FepA also appears capable of transporting DHBA and DHBS. Interestingly, DHBA-iron complexes were used as iron sources not only by *H. parainfluenzae* and *H. paraphrophilus*, but also by *H. influenzae* type b strain Eagan, which lacks the enterobactin receptor and does not induce major IROMPs. Similar results were also obtained with other *H. influenzae* strains (data not shown). Thus iron complexed with DHBA may be utilized by more than one mechanism in *Haemophilus*. Furthermore, each of the haemophili examined was also capable of using iron

chelated by citrate and nitrilotriacetate, suggesting that they may possess an efficient, but as yet unidentified mechanism(s), for mediating either direct uptake or iron release from these compounds. In this respect they resemble the neisseriae, which have also been reported to assimilate a variety of iron chelates including iron citrate, oxalate, nitrilotriacetate and pyrophosphate (Mickelsen & Sparling, 1981).

Thus, haemophili express multiple iron-uptake mechanisms which may be either siderophore-independent, as illustrated by the acquisition of transferrin-bound iron by *H. influenzae* (Morton & Williams, 1990), or siderophore-dependent, as demonstrated by the utilization of enterobactin by *H. parainfluenzae* and *H. paraphrophilus*. However, the more pathogenic *H. influenzae* appears to possess the advantage in extracellular body fluids of being able to sequester iron tightly bound to a variety of iron and haem-binding proteins.

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