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# Expression of the *Haemophilus influenzae* Transferrin Receptor Is Repressible by Hemin but Not Elemental Iron Alone

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**The absolute requirement for elemental iron and the porphyrin nucleus for growth of *Haemophilus influenzae* led us to investigate the role of iron and hemin in regulation of expression of the *H. influenzae* transferrin receptor. *H. influenzae* type b strain HI689 was grown in brain heart infusion broth supplemented with β-NAD and either 10 or 0.1 μg of hemin $ml^{-1}$. Transferrin-binding ability was determined with a dot blot assay using human transferrin-horseradish peroxidase conjugate. Cells grown in media with 0.1 μg of hemin $ml^{-1}$ bound transferrin, but organisms grown in media with 10 μg $ml^{-1}$ did not. In hemin-restricted media, transferrin binding occurred despite addition of up to 10 mM ferric nitrate, ferric citrate, or ferric $PP_i$, whereas addition of 10 μg of hemoglobin $ml^{-1}$ repressed expression. The breadth of species distribution of this mode of regulation was determined with strains previously characterized by multilocus enzyme electrophoresis. When grown in hemin-restricted media, 24 of 28 type b strains and 52 of 57 serologically nontypeable strains exhibited transferrin binding, although none did so in hemin- and iron-sufficient media. Strain HI689 and serologically nontypeable strain HI1423 grown in heat-inactivated pooled normal human serum, human cerebrospinal fluid, or human breast milk exhibited transferrin binding. Growth in these fluids with 10 μg of added hemin $ml^{-1}$ abolished transferrin binding, whereas addition of 10 mM ferric nitrate did not. These data suggest that the transferrin receptor of *H. influenzae* is regulated by levels of hemin but not elemental iron alone and that this property is widely distributed among several major cloned families in the species.**

Transferrins are iron-binding glycoproteins found in mammalian extracellular fluids (3). Under normal conditions, the concentration of human transferrin in serum is about 30 μM and the transferrin is 30% saturated with iron. The level of free iron in serum is approximately $10^{-18}$ M, a concentration well below that required to maintain bacterial growth (6). During infection, iron bound to transferrin decreases and intracellular stores increase, a process which further restricts the amount of iron available to an invading organism (32).

Many gram-negative pathogens overcome iron restriction by expressing high-affinity iron uptake systems comprising low-molecular-weight iron chelators (siderophores) and their corresponding outer membrane protein receptors (6). These uptake mechanisms are regulated by the level of intracellular elemental iron (2, 6). Other pathogens, such as *Neisseria gonorrhoeae* (13), *Neisseria meningitidis* (25), and possibly *Bordetella pertussis* (1, 14, 22) and *Bordetella bronchiseptica* (14), express a siderophore-independent iron uptake mechanism which requires direct contact between the bacterial cell and the host iron-binding protein. The siderophore-independent systems appear to be regulated by elemental iron levels, since binding of transferrins is induced by growth in iron-restricted media and repressed by addition of elemental iron (22, 25, 27).

*Haemophilus influenzae* colonizes the upper respiratory tract of up to 80% of children and adults (17). Among these strains, *H. influenzae* type b has been the most important human pathogen and is a major cause of invasive infections such as childhood meningitis and epiglottitis (31). Unencapsulated strains of *H. influenzae* are also pathogenic in humans, frequently causing otitis media in children and pneumonia in adults (5, 31).

*H. influenzae* does not produce siderophores; however, it acquires transferrin-bound iron by a mechanism involving direct transferrin binding at the bacterial cell surface (15, 16, 26). Expression of transferrin-binding proteins of *H. influenzae* can be induced by growth in media iron restricted by addition of 100 μM ethylenediamine-di-*ortho*-hydroxyphenylacetic acid (EDDA) with 0.5 or 2 μg of protoporphyrin IX (PPIX) $ml^{-1}$ added as a porphyrin source. Growth in media supplemented with PPIX but with no EDDA or in media with PPIX, EDDA, and $Fe(NO_3)_3$ (to overcome EDDA-induced iron restriction) does not result in expression of transferrin-binding proteins (15, 26). These data suggest that transferrin binding in *H. influenzae* is iron regulated. However, in the iron-restricted media described above, the organism is starved for both iron and hemin. Although adequate PPIX to satisfy requirements for the porphyrin nucleus is supplied, iron is not available for insertion into the nucleus to form hemin; thus, the organism is effectively starved for hemin. The goal of our investigations was to characterize the regulation of transferrin binding by *H. influenzae*.

## MATERIALS AND METHODS

**Bacterial strains.** Strains of *H. influenzae* were previously characterized by the electrophoretic mobility of 15 metabolic enzymes (18). Ninety-four distinct electrophoretic types (ETs) were distinguished. Twenty-nine of the ETs represent type b strains and occur in four clusters designated A through D; the remaining ETs represent serologically nontypeable strains. Eighty-five strains representing 85 different ETs were used in this study; 28 of them were type b strains,

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and 57 of them were serologically nontypeable. *Escherichia coli* JM109 was used where indicated.

**Growth conditions.** *H. influenzae* was grown overnight in brain heart infusion broth (BHI) (Difco, Detroit, Mich.) supplemented with 10 μg each of hemin and β-NAD (supplemented BHI [sBHI]) $ml^{-1}$ or BHI supplemented with 0.1 μg of hemin and 10 μg of β-NAD (hemin-restricted BHI [hrBHI]) $ml^{-1}$. sBHI is replete for both hemin and iron, whereas hrBHI is replete for elemental iron but restricted for hemin. In certain experiments hrBHI was additionally supplemented with 1 or 10 mM ferric nitrate, ferric citrate, or ferric $PP_i$ or 10 μg of human hemoglobin (Sigma, St. Louis, Mo.) $ml^{-1}$. For iron-restricted growth, *H. influenzae* was grown in BHI supplemented with 100 μM EDDA and 10 μg of PPIX (iron restricted BHI [irBHI]) $ml^{-1}$, and in some experiments irBHI was additionally supplemented with 10 μg of hemin $ml^{-1}$, giving a medium which is restricted for elemental iron and replete for hemin.

For other experiments, *H. influenzae* was grown for 3 h in heat-inactivated (65°C for 10 min) pooled normal human serum, human cerebrospinal fluid (CSF), or human breast milk. Pooled human breast milk was centrifuged at 56,000 × *g* for 90 min; the fluid was separated from the fat and filter sterilized prior to use (7). In some experiments, these fluids were supplemented with either 10 mM ferric nitrate or 10 μg hemin $ml^{-1}$ prior to growth of the bacteria.

*E. coli* was grown in Luria-Bertani broth (LB) overnight. In some experiments, 1 mM ferric nitrate was added to the medium. Iron restriction was achieved by addition of 500 μM EDDA.

**Transferrin-binding assays.** Transferrin-binding assays were performed with dot blots, as previously described (15, 16). Briefly, bacteria were harvested by centrifugation, resuspended to $10^9$ organisms $ml^{-1}$ in 0.9% (wt/vol) NaCl–10 mM Tris-HCl, pH 7.4 (TBS), and 5-μl aliquots were applied to nitrocellulose membranes in a dot blot manifold (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Membranes were removed, air dried, blocked with 0.5% skim milk in TBS for 1 h, and incubated with 500 ng of human transferrin-horseradish peroxidase conjugate (Jackson ImmunoResearch, West Grove, Pa.) $ml^{-1}$ in TBS for 1 h prior to development with 25 μg of 4-chloro-1-naphthol $ml^{-1}$–0.01% (vol/vol) $H_2O_2$ in 10 mM Tris-HCl, pH 7.4.

In some experiments, bacteria grown in media supplemented with additional iron up to 10 mM were washed extensively prior to the dot blot assay. Organisms were washed by one of the following five protocols: (i) 3 washes in 10-fold excess TBS, (ii) 5 washes in 10-fold excess TBS, (iii) 10 washes in 10-fold excess TBS, (iv) 5 washes in 10-fold excess TBS followed by 5 washes in 10-fold excess 100 μM EDDA in TBS, or (v) 5 washes in 10-fold excess TBS followed by 5 washes in 10-fold excess 1 mg of desferoxamine (CIBA, Summit, N.J.) $ml^{-1}$ in TBS.

## RESULTS

*H. influenzae* HI689, a type b strain assigned to ET cluster B (18), bound transferrin when grown in hrBHI; when grown in sBHI, HI689 did not bind transferrin (Fig. 1). The concentrations of exogenous hemin and elemental iron in sBHI are sufficient to satisfy the requirements of the organism for hemin and iron; in hrBHI, elemental iron levels are unchanged from those in sBHI but hemin levels are restricted. In addition, strain HI689 grown in irBHI bound transferrin but did not bind transferrin when grown in irBHI supplemented with hemin (data not shown). The data suggest that

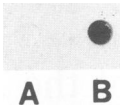


FIG. 1. Transferrin-binding dot blot assay of *H. influenzae* type b strain HI689 grown in hemin-replete (A) or hemin-restricted (B) medium, fixed onto nitrocellulose membranes, and probed with transferrin-horseradish peroxidase conjugate.

hemin, but not elemental iron alone, represses expression of transferrin-binding activity.

To further characterize the regulation of transferrin binding, dot blot assays were performed with strain HI689 grown in hrBHI supplemented either with 10 mM iron as ferric nitrate, ferric citrate, or ferric $PP_i$ or with human hemoglobin (10 μg $ml^{-1}$). Transferrin binding was expressed following growth in hrBHI supplemented with each of the elemental iron sources but not following supplementation with hemoglobin (Fig. 2). It is possible that growth in such high levels of iron, vastly in excess of those necessary to support growth, could result in nonspecific binding of large amounts of iron to the cell surface; if transferrin bound to this surface-associated iron, a false-positive response might result. In order to eliminate this possibility, strain HI689 grown in hrBHI with either 1 or 10 mM added ferric nitrate was washed extensively with TBS or with TBS containing one of the iron-binding compounds (EDDA and desferoxamine) prior to dot blot assays. Cells washed by each of the protocols outlined above bound transferrin, as did unwashed cells. In addition, HI689 grown in sBHI with 1 mM added ferric nitrate did not bind transferrin, even when it had not been washed. *E. coli* did not bind transferrin following growth in LB, iron-restricted LB, or LB with 1 mM ferric nitrate, even after the least rigorous wash conditions. These data indicate that the observed transferrin binding is a specific interaction between *H. influenzae* and transferrin and not the result of nonspecific binding to cell-associated iron.

To determine the breadth of species distribution of transferrin receptor expression in hrBHI, 84 additional genetically characterized *H. influenzae* strains were tested (Fig. 3). No strain bound transferrin when grown in sBHI. All serotype b isolates assigned to ET clusters A, B, and C bound transferrin when they were grown in hrBHI; however, none of the four strains in the type b cluster D bound transferrin under these growth conditions. Of the serologically nontypeable strains tested, 52 of 57 bound transferrin when they were grown in hrBHI. Those which were negative in our assay represented genetically highly divergent organisms (Fig. 3).

The potential in vivo regulation of transferrin binding was

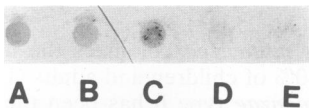


FIG. 2. Transferrin-binding dot blot assay of *H. influenzae* type b strain HI689 grown in a hemin-restricted medium (hrBHI) with added 10 mM ferric citrate (A), hrBHI with added 10 mM ferric nitrate (B), hrBHI with added 10 mM ferric $PP_i$ (C), hrBHI with 10 μg of hemoglobin $ml^{-1}$ added (D), or a hemin-replete medium (E), fixed to nitrocellulose membranes, and probed with transferrin-horseradish peroxidase conjugate.

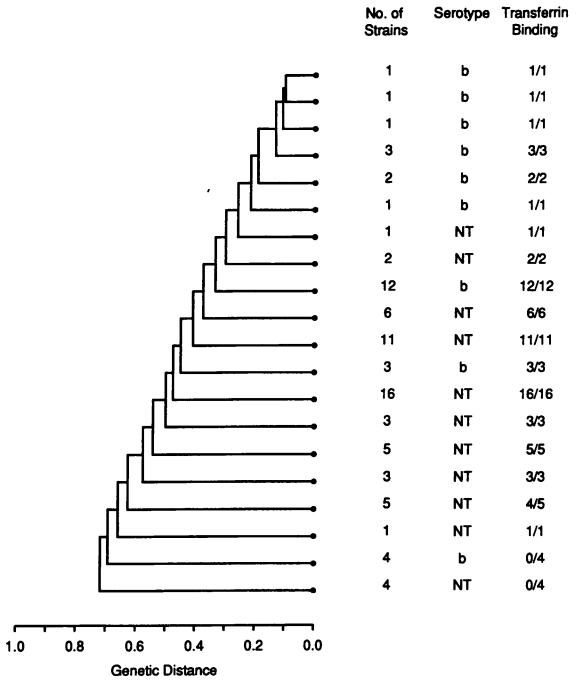


FIG. 3. Dendrogram showing the genetic relationship and distribution of transferrin binding among 85 ETs of *H. influenzae*. Transferrin binding was assayed as described in the Materials and Methods section by using human transferrin-horseradish peroxidase conjugate as a probe. Each dot represents a strain or a group of closely related strains. Dots (from top to bottom) represent ETs 1; 3; 4; 5 to 7; 8 and 9; 10; 11; 12 and 13; 14 to 25; 26, 27, and 29 to 32; 35 to 45; 46 to 48; 49 to 51, 53 to 55, 57 to 62, and 64 to 67; 68 to 70; 71 to 75; 77 to 79; 80 to 84; 85; 87 to 90; and 91 to 94 (18). Type b strains were assigned to four clusters based on ET (18), designated A through D, and represented, respectively, by dots 1 to 6, 9, 12, and 19. NT, nontypeable.

investigated by incubating *H. influenzae* in human body fluids. Growth of strain HI689 in breast milk, heat-inactivated pooled normal human serum, and human CSF resulted in bacteria which bound transferrin (Table 1). Supplementation with hemin prior to growth abolished binding in all cases. However, addition of ferric nitrate did not affect binding. Similarly, growth of *H. influenzae* HI1423, a serologically nontypeable strain representing ET 83, in each of the three body fluids resulted in transferrin binding in the dot blot assay. This binding was abolished during growth in human body fluids containing 10 μg of hemin $ml^{-1}$ but was unaffected by addition of 10 mM ferric nitrate to the fluids.

TABLE 1. Transferrin binding by *H. influenzae* type b strain HI689 and nontypeable strain HI1423[a]

| Medium | Transferrin binding[b] | |
|---|---|---|
| | HI689 | HI1423 |
| CSF | + | + |
| CSF plus hemin, 10 μg $ml^{-1}$ | − | − |
| CSF plus $Fe(NO_3)_3$, 10 mM | + | + |
| Serum | + | + |
| Serum plus hemin, 10 μg $ml^{-1}$ | − | − |
| Serum plus $Fe(NO_3)_3$, 10 mM | + | + |
| Breast milk | + | + |
| Breast milk plus hemin, 10 μg $ml^{-1}$ | − | − |
| Breast milk plus $Fe(NO_3)_3$, 10 mM | + | + |

[a] Results of assays after growth for 3 h in normal human CSF, heat-inactivated pooled normal human serum, or heat-inactivated normal human breast milk either with no additions or supplemented as specified. Transferrin binding was assayed by dot blot with human transferrin-horseradish peroxidase conjugate.

[b] +, positive; −, negative.

## DISCUSSION

Many gram-negative bacteria induce production of low-molecular-weight iron chelators (siderophores) in response to iron limitation. In *E. coli* these systems are regulated by the *fur* gene product, a protein of 17 kDa which functions as a repressor. When bound to its corepressor, elemental iron, the Fur protein binds to the operator sequence of the operon, preventing transcription. Many systems in a wide range of gram-negative bacteria are apparently regulated by mechanisms closely related to the *fur* system (11, 20, 23, 24, 29).

In organisms which utilize transferrin-bound iron via a direct interaction with the glycoprotein, the mechanism of regulation has not been defined. Transferrin binding in species of the family *Neisseriaceae* and in *Bordetella* species is inducible by growth in iron-restricted media and repressible by addition of an elemental iron source (14, 25, 27), suggesting that transferrin binding by these species is regulated by intracellular iron levels, possibly via a mechanism analogous to Fur (2).

The data presented in this study suggest that transferrin binding in *H. influenzae* is regulated by the level of hemin and not elemental iron alone. Expression of the transferrin receptor is induced by growth in hemin-restricted media and repressed by addition of excess hemin but not by the addition of elemental iron up to 10 mM. Transferrin binding was also repressible by addition of hemoglobin, which fulfills both the hemin and iron requirements of *H. influenzae* (19, 30, 33). In addition, the transferrin receptor was detected after growth of strains in human body fluids, in which levels of both hemin and free elemental iron are restricted. Transferrin binding was repressed by addition of hemin to the body fluids but not by addition of iron up to 10 mM, a level more than sufficient to saturate iron-binding proteins present in the fluids.

Previous studies indicated that transferrin binding in *H. influenzae* is inducible by iron starvation and repressible by addition of elemental iron (16, 26). In these cases, the method of iron starvation was addition of 100 μM EDDA and 0.5 or 2 μg of PPIX $ml^{-1}$. Although an adequate porphyrin nucleus was provided in these experiments, iron is not available for insertion into the porphyrin molecule to form hemin; therefore, these conditions also result in hemin starvation. Addition of iron to these restricted media resulted in loss of transferrin-binding activity; however, addition of iron relieves the starvation of both elemental iron and hemin. Our data demonstrate that when hemin levels are restricted but additional elemental iron is added, transferrin binding is not repressed, indicating that elemental iron is not the crucial molecule in regulation of the receptor.

Other systems in *H. influenzae* may also be repressible by hemin. Coulton and Pang reported that growth in hemin-restricted media resulted in enhanced production of a 43-kDa protein (4), and Stull reported induction of a 38-kDa outer membrane protein after growth in hemin-limiting conditions (30). Replacement of hemin in iron-restricted growth media with PPIX resulted in expression of increased amounts of an 84-kDa polypeptide and expression of two outer membrane

proteins of 120 and 150 kDa in strains of serologically nontypeable *H. influenzae* (12). A recently reported hemin-human hemopexin complex-binding protein in *H. influenzae* type b was induced following growth in hemin-restricted, iron-replete conditions (8). A hemin-binding outer membrane protein of 39.5 kDa was isolated from *H. influenzae* type b cells grown in an iron-restricted medium (10). Iron restriction was achieved by addition of EDDA in the presence of PPIX, effectively starving for hemin, which raised the possibility that this protein is regulated by hemin levels. Recently, we have also demonstrated that the expression of human hemoglobin binding by *H. influenzae* is induced by growth in hemin-restricted, iron-replete media (9). Thus, hemin may be important in regulating the iron and hemin uptake systems of *H. influenzae*. This potential regulatory mechanism appears to be widely distributed throughout the species *H. influenzae*, including both type b and serologically nontypeable strains, although several highly divergent type b and nontypeable strains did not bind transferrin following growth in hrBHI. It is likely that some isolates classified as *H. influenzae* biotype IV diverge from other strains to such a degree that they may require classification as a separate species (18, 21). All of the strains which did not bind transferrin were, with a single exception, biotype IV strains. Hemin restriction is likely to induce iron and hemin uptake systems in *H. influenzae* types a, c, d, e, and f, since these strains (when grown in hemin-restricted iron-replete media) all bound human hemoglobin (9).

We speculate that the *H. influenzae* transferrin receptor is regulated by a mechanism analogous to the *fur* system described for *E. coli* (2) but in which the corepressor is hemin and not iron. However, this regulatory mechanism may be more complex than the *fur* system. We have used a novel mutagenesis method (28) to construct mutants which constitutively express transferrin-binding activity. However, hemoglobin binding in these mutants remained hemin repressible (unreported observations), possibly indicating that the two phenotypes are independently regulated despite regulation being mediated in both cases by the same molecule. Work to elucidate this potentially novel regulatory mechanism and to define the genes and gene products involved is continuing.

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