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# Iron Acquisition in *Pasteurella haemolytica*: Expression and Identification of a Bovine-Specific Transferrin Receptor

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**Seven type 1 field isolates of *Pasteurella haemolytica* were screened for their ability to use different transferrins as a source of iron for growth. All seven strains were capable of using bovine but not human, porcine, avian, or equine transferrin. A screening assay failed to detect siderophore production in any of the strains tested. Iron-deficient cells from these strains expressed a binding activity, specific for bovine transferrin, that was regulated by the level of iron in the medium. Inhibition of expression by translation and transcription inhibitors suggested that iron regulation was occurring at the gene level. Affinity isolation of receptor proteins from all seven strains with biotinylated bovine transferrin identified a 100-kilodalton iron-regulated outer membrane protein as the bovine transferrin receptor. Iron-regulated outer membrane proteins of 71 and 77 kilodaltons were isolated along with the 100-kilodalton protein when less stringent washing procedures were employed in the affinity isolation procedure.**

Bovine pneumonic pasteurellosis, or shipping fever, continues to be a major cause of economic loss to the cattle industry (19). The disease in cattle is characterized pathologically by a lobar pneumonia with a predominantly fibrinous exudate and is commonly associated with pleuritis (37). The disease is usually associated with biotype A, serotype 1 strains of *Pasteurella haemolytica* (3) and occasionally with biotype A, serotype 2 strains (36). Relatively little is known about the pathogenesis of pneumonic pasteurellosis (23) or about virulence factors of the organism (18). This lack of knowledge is reflected by the failure of current vaccines, which have not only been inconsistent in reducing the incidence of the disease (7, 13) but in some cases have increased the severity (34).

The limitations of current vaccines have prompted a search for a variety of components in *P. haemolytica* which may serve as immunogens. These include bacterial cell walls and capsular materials (11), a carbohydrate-protein subunit (17), inner and outer membrane proteins (31), and a lipopolysaccharide with endotoxic properties (8). Some of these have been found to possess immunogenic properties of limited protective value. An exotoxin (leukotoxin) toxic to ruminant leukocytes (4) has also been suggested as an important virulence factor in the disease process (30).

Studies with other bacterial pathogens have established that the ability to acquire iron in vivo is an important virulence factor (32). Little is known about iron acquisition during *P. haemolytica* infection, although previous studies suggest that iron acquisition may be important in the disease process. For example, the production of the virulence factor leukotoxin requires iron-containing compounds such as hemoglobin, transferrin, or lactoferrin (14). Also, experimental infection in mice by *P. haemolytica* is promoted by iron (1) and by hemoglobin (6).

High-affinity iron uptake systems capable of removing iron for growth in vivo from compounds such as transferrin and lactoferrin have been studied in a number of bacterial species (9, 22, 24, 25). Two basic types of system have been demonstrated: one type mediated by small chemical iron chelators called siderophores and a second type involving direct binding of transferrin or lactoferrin. Both systems involve the production of iron-repressible outer membrane proteins. The type of iron acquisition system in *P. haemolytica* is not known.

In a recent study Deneer and Potter (10) observed that several iron-repressible outer membrane proteins with apparent molecular weights of 71,000, 77,000, and 100,000 were induced in *P. haemolytica* in response to iron limitation. Analysis of the iron-repressible outer membrane proteins showed that they reacted only with convalescent antisera from *P. haemolytica*-infected calves, suggesting that they were expressed in vivo. The role of these proteins in iron acquisition has not been determined.

This study was initiated to aid in defining the mechanism by which *P. haemolytica* acquires iron. We report here that *P. haemolytica* is capable of obtaining iron for growth from bovine transferrin but not from human, porcine, equine, or avian transferrin, which correlates with specific binding of bovine transferrin by *P. haemolytica* cells or membranes. We also demonstrate that the transferrin-binding activity is regulated by the level of iron in the medium and that induction of receptor expression in *P. haemolytica* is regulated by iron at the transcription level. In addition, we demonstrate by affinity isolation of bovine transferrin receptor proteins that the 100-kilodalton (kDa) iron-repressible outer membrane protein described by Deneer and Potter (10) is in fact a receptor protein for bovine transferrin and that the receptor is readily isolated in all the strains tested.

## MATERIALS AND METHODS

**Bacterial strains.** *P. haemolytica* PH21, PH24, PH27, and PH45, all serotype A1, were field isolates provided by A. Potter of the Veterinary Infectious Disease Organization, Saskatoon, Saskatchewan. Strains h44, h45, and h46 were clinical serotype A1 isolates from calves with fibrinous pneumonia generously provided by S. Lundberg, Veterinary Laboratory, Regional Agricultural Building, Airdrie, Alberta. *Actinobacillus pleuropneumoniae* h49, serotype 7, was a disease isolate from swine and was provided by S. Lundberg. *Neisseria meningitidis* B16B6 (serogroup B, se-

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rotype 2a) was originally obtained from C. Frasch, Bethesda, Md. *Pseudomonas cepacia* 715j, a clinical isolate from a patient with cystic fibrosis was obtained from Donald Woods, Department of Microbiology and Infectious Diseases, University of Calgary, Alberta.

**Growth conditions.** All bacterial strains were stored frozen at −70°C in 30% glycerol. Isolates from the frozen stocks were streaked onto chocolate agar plates and incubated for 18 to 24 h at 37°C in a 5% $CO_2$ atmosphere. Iron-restricted growth was achieved by growing bacteria in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) supplemented with the iron chelator ethylenediaminedihydroxyphenylacetic acid (EDDA; Sigma Chemical Co., St. Louis, Mo.) or, in some experiments, with 2,2-dipyridyl (Sigma) or desferrioxamine mesylate (Sigma) to a final concentration of 100 μM. Iron-rich broth was prepared by adding $FeCl_3$ (final concentration, 120 μM) to the BHI-EDDA broth.

In the growth experiment, an exponential-phase culture in BHI-EDDA broth was diluted to an $A_{600}$ of 0.05 in fresh BHI-EDDA broth. Samples of 50 μl of the diluted culture were evenly spread on the surfaces of dry, freshly prepared BHI plates containing 100 μM EDDA. Blank 0.25-in. (ca. 0.625-cm)-diameter antibiotic disks (Difco) impregnated with the respective iron-loaded transferrin, bovine hemoglobin (Sigma) or with $FeCl_3$ were placed onto the surface of the plates and the latter were incubated in a 5% $CO_2$ atmosphere overnight.

In expression-inhibition experiments, antibiotics at the indicated concentrations were added to exponential-phase cultures in BHI (final $A_{600}$ of 0.05) 10 min before the addition of 100 μM EDDA.

For large-scale cultures used in membrane preparations, cells from overnight cultures on chocolate agar plates were used to inoculate starter cultures of BHI broth. After incubation for 2 to 3 h at 37°C, the cells were used to inoculate 1.5-liter cultures of broth with or without 100 μM EDDA to a starting $A_{600}$ of 0.05, reincubated at 37°C, and harvested after 4 to 5 h of incubation.

**Screening for siderophore production.** The media and method used to screen for siderophore production were essentially as described by Schwyn and Neilands (29).

**Preparation of transferrins and derivatives.** Human, bovine, chicken, and horse transferrins were obtained from Sigma, and porcine transferrin was obtained from The Binding Site Ltd., Birmingham, England. Iron loading of commercial apotransferrins to 30 or 100% saturation was performed as described previously (15). Biotinylation of the apo form of bovine and porcine transferrins with *N*-hydroxysuccinimide biotin was performed essentially as described previously (27). A horseradish peroxidase (HRP) conjugate of human transferrin (HRP-hTf) was obtained from Jackson Immunoresearch Laboratories, Avondale, Pa. The HRP conjugates of bovine and porcine transferrins (HRP-bTf and HRP-pTf, respectively) were prepared as described by Wilson and Nakane (35). After chemical conjugation, the mixture of HRP and transferrin was subjected to gel filtration on a Spherogel TSK 3000 SW high-performance liquid chromatography column (Beckman Canada, Mississauga, Ontario). The fractions from the peak corresponding to a 1:1 conjugate were pooled and dialyzed, and samples were frozen and stored at −70°C.

**Transferrin binding assay.** The solid-phase binding assay for bovine transferrin was essentially a modification of the procedure previously described for detecting human transferrin binding (28). After the cells or membranes were spotted onto HA paper (Millipore Corp., Bedford, Mass.) and blocked in 0.5% skim milk, the paper was exposed to blocking solution containing 450 μg of HRP-bTf, HRP-hTf, or HRP-pTf per ml. In competition experiments, the indicated amounts of unconjugated protein (100% Fe saturated) was included in the binding mixture. The incubation times, washing steps, and development with HRP-substrate mixture were performed essentially as described previously (28).

**Preparation of outer membranes.** Crude membranes from iron-rich and iron-poor cells were prepared as previously described (27). Outer membranes were prepared by selective detergent extraction of the crude membranes as previously described (27).

**Affinity isolation of transferrin receptor proteins.** Isolation of the *P. haemolytica* bovine transferrin receptor was performed essentially as previously described for the *N. meningitidis* human transferrin receptor (27), except that biotinylated bovine transferrin was used in place of biotinylated human transferrin and outer membranes were used in place of total membranes. After the final washing step, the samples were suspended in 100 to 200 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer without β-mercaptoethanol and boiled for 5 min. This was followed by rapid cooling on ice and then centrifugation at 750 × *g* for 5 min. The supernatant was transferred to a separate tube, and β-mercaptoethanol was added to a final concentration of 1.4 M. A 25 to 50 μl portion of this sample was applied to an SDS-PAGE (10% polyacrylamide) gel. SDS-PAGE and silver staining of gels were performed as described previously (27).

## RESULTS

**Use of iron-containing compounds for growth.** To test the ability of strains of *P. haemolytica* to use different protein sources of iron for growth, the simple plate assay described in Materials and Methods was utilized (Fig. 1). Bovine transferrin and ferric chloride stimulated the growth of *P. haemolytica* h44, whereas no growth was observed around disks containing porcine or human transferrin (Fig. 1A). The fact that *N. meningitidis* and *A. pleuropneumoniae* could use human and porcine transferrins, respectively (Fig. 1B and C), indicates that the failure of *P. haemolytica* h44 to use these protein sources was not due to any inadequacies in their preparation. Similar experiments demonstrated that all of the type A1 strains of *P. haemolytica* examined were able to use bovine transferrin, but not transferrins from other animal species, as a source of iron for growth (Table 1).

**Assessment of siderophore production.** To determine whether strains of *P. haemolytica* possessed siderophore-mediated iron acquisition systems, a simple screening assay for siderophore production (29) was performed. None of the *P. haemolytica* type A1 strains examined was able to produce siderophore on chrome azurol S medium. Under the same experimental conditions, the positive control strain *P. cepacia* 715j produced siderophores, whereas the negative control strain *N. meningitidis* B16B6 failed to do so.

**Detection and regulation of expression of transferrin-binding activity.** The exclusive use of bovine transferrin for growth observed in *P. haemolytica* coupled with its lack of siderophore production suggested to us that, by analogy with *N. meningitidis*, iron acquisition would be mediated by a surface receptor. We thus used a simple binding assay, analogous to that used previously for *N. meningitidis* (28), that required the labeling of bovine transferrin with HRP. Using the HRP-bTf prepared as described above, we per-

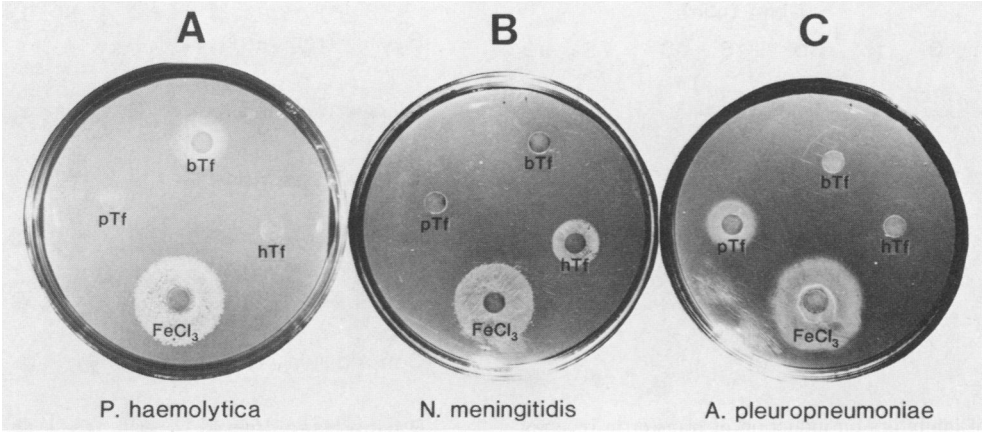


FIG. 1. Plate bioassay for growth stimulation of *P. haemolytica* type A1 strain h44 (A) by various iron compounds. Organisms were seeded onto BHI agar containing 100 μM EDDA, filters with the indicated iron sources were applied, and the plates were incubated as described in Materials and Methods. Disks contained (from top clockwise) 30% Fe-saturated bovine transferrin (bTf, 2.5 nmol), 30% Fe-saturated human transferrin (hTf; 2.5 nmol), $FeCl_3$ (250 nmol), and 30% Fe-saturated porcine transferrin (pTf; 2.5 nmol). *N. meningitidis* (B) and *A. pleuropneumoniae* (C) served as controls.

formed binding assays with cells of *P. haemolytica*, *A. pleuropneumoniae*, and *N. meningitidis* that were iron starved in BHI containing 100 μM EDDA. The use of iron-starved cells was prompted by the observation that transferrin-binding activity was iron repressible in *N. meningitidis* (28). In these experiments, binding of HRP-bTf to cells of *P. haemolytica* but not to cells of *N. meningitidis* or *A. pleuropneumoniae* was observed (data not shown). Also, no binding of *P. haemolytica* cells occurred when HRP-hTf or HRP-pTf was substituted for HRP-bTf (data not shown).

To determine whether the bovine transferrin-binding activity was iron regulated, *P. haemolytica* h44 was grown in broth containing the iron chelator EDDA, desferrioxamine, or 2',2-dipyridyl. Growth on any of these chelators resulted in significant levels of detectable bovine transferrin-binding activity (Fig. 2). When excess $FeCl_3$ was added to the chelator-containing broth, no bovine transferrin binding was detected (Fig. 2), demonstrating that the induction of receptor expression by the chelators was due to the effective withholding of iron.

The results in Fig. 2 clearly illustrate iron regulation of transferrin-binding activity but provide no information as to the mechanism or level at which regulation was occurring. In an attempt to provide more information on the kinetics of expression of the receptor-binding activity and the effects of transcription and translation inhibitors, the experiment illustrated in Fig. 3 was performed. In the absence of iron-chelators (row 1), there was little or no expression of transferrin-binding activity. Addition of EDDA induced the expression of binding activity in detectable amounts within 30 min and reached near-maximal levels within 60 to 90 min

TABLE 1. Growth of strains of *P. haemolytica* with different iron sources[a]

| Species and strain (serotype) | Growth on the following protein source: | | | | | | |
|---|---|---|---|---|---|---|---|
| | bTf | bHb | hTf | pTf | eTf | cTf | $FeCl_3$ |
| *P. haemolytica* | | | | | | | |
| h44 (type 1)[b] | + | + | − | − | − | − | + |
| h45 (type 1)[b] | + | + | − | − | − | − | + |
| h46 (type 1)[b] | + | + | − | − | − | − | + |
| PH21 (type 1)[c] | + | + | − | − | − | − | + |
| PH24 (type 1)[c] | + | + | − | − | − | − | + |
| PH27 (type 1)[c] | + | + | − | − | − | − | + |
| PH45 (type 1)[c] | + | + | − | − | − | − | + |
| *N. meningitidis* B16B6 | − | NT | + | − | − | − | + |
| *A. pleuropneumoniae* h92 | − | NT | − | + | − | − | + |

[a] Growth experiments were performed as described in Materials and Methods and illustrated in Fig. 1. bTf, hTf, pTf, eTf, and cTf, Bovine, human, porcine, equine, and chicken transferrins, respectively; bHb, bovine hemoglobin; NT, not tested.
[b] Strains obtained from Alberta Agriculture.
[c] Strains obtained from the Veterinary Infectious Disease Organization.

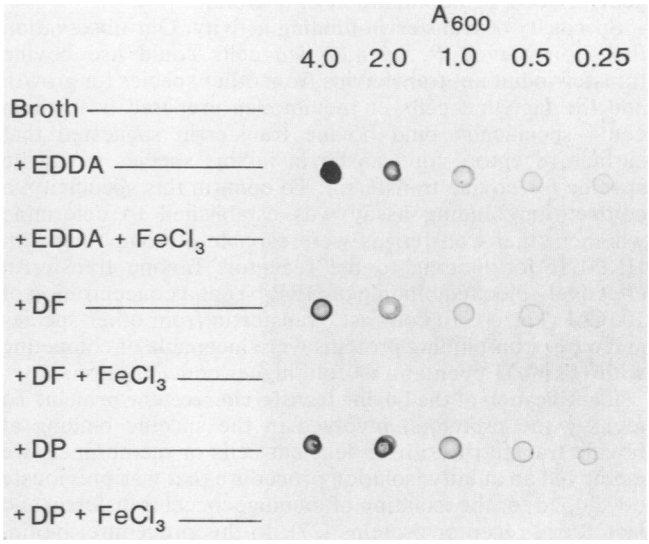


FIG. 2. Effect of iron on expression of transferrin-binding activity in *P. haemolytica* h44. Intact bacterial cells grown under the indicated conditions were resuspended to the indicated $A_{600}$, and 2 μl of each suspension was spotted onto the paper. After drying and blocking, the paper was exposed to HRP-bTf before washing and development with an HRP-substrate mixture as described in Materials and Methods. Growth conditions: +EDDA, 100 μM EDDA; +EDDA + $FeCl_3$, 100 μM EDDA and 120 μM $FeCl_3$; +DF, 100 μM desferrioxamine; +DF + $FeCl_3$, 100 μM desferrioxamine and 120 μM $FeCl_3$; +DP, 100 μM dipyridyl; +DP + $FeCl_3$, 100 μM dipyridyl and 120 μM $FeCl_3$.

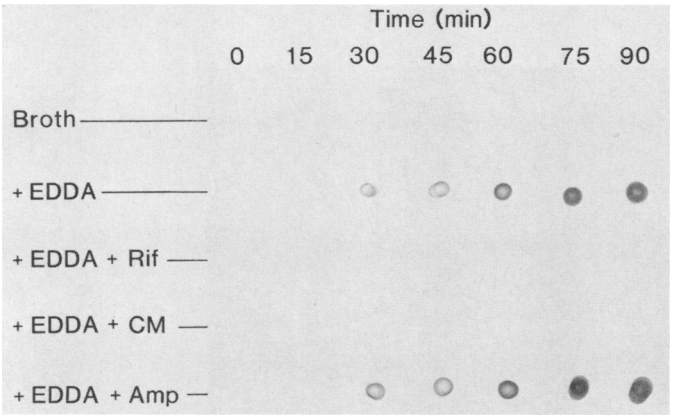


FIG. 3. Effect of inhibitors on induction of transferrin receptor expression. *P. haemolytica* h44 was subcultured from a log-phase culture in BHI broth to an initial $A_{600}$ of 0.05 in BHI broth containing the indicated additions. Samples taken at the indicated time periods were centrifuged, and the cells were suspended and spotted onto HA paper. After dying and blocking, the paper was incubated with HRP-bTf, washed, and developed with substrate mixture as described in Materials and Methods. Growth conditions: +EDDA, 100 μM EDDA; +EDDA + Rif, 100 μM EDDA and 20 μg of rifampin per ml; +EDDA + CM, 100 μM EDDA and 20 μg of chloramphenicol per ml; +EDDA + AMP, 100 μM EDDA and 20 μg of ampicillin per ml.

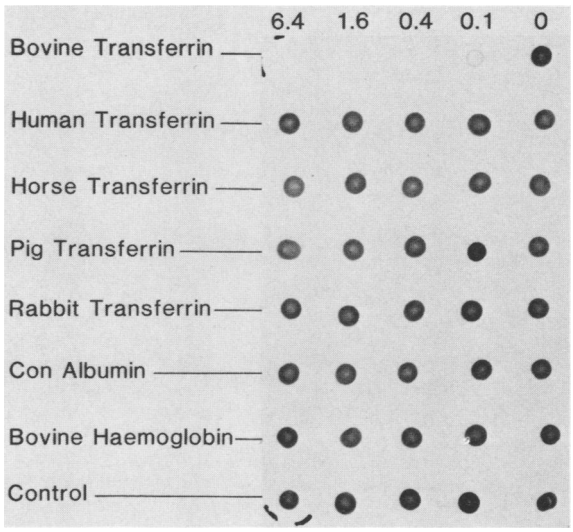


FIG. 4. Specificity of transferrin receptor in *P. haemolytica* h44. Intact iron-deficient cells from strain h44 were spotted onto HA paper and exposed to mixtures of HRP-bTf and the indicated concentrations (micromolar) of 100% Fe-saturated unconjugated proteins in the microdot apparatus. After incubation with the binding mixtures, the paper was washed and exposed to HRP-substrate mixture for color development. Con Albumin, Chicken ovotransferrin.

after EDDA addition (Fig. 3). This expression was inhibited by chloramphenicol, suggesting that the expression is not posttranslation, and by rifampin, suggesting that the control of expression is at the transcription level. The inability of ampicillin to inhibit binding activity would suggest that the inhibition by rifampin and chloramphenicol was not due to a general nonspecific inhibition of bacteria.

**Specificity of transferrin-binding activity.** Our observation that iron-starved *P. haemolytica* cells could use bovine transferrin but not transferrins from other species for growth and the fact that cells or membranes prepared from them could specifically bind bovine transferrin suggested that surface receptors for transferrin in this species would be specific for bovine transferrin. To confirm this specificity, a competition binding assay was established to determine whether other transferrins were capable of competing with HRP-bTf for binding to the receptor. Bovine transferrin effectively blocked binding of HRP-bTf at a concentration of 100 nM (Fig. 4). In contrast, transferrin from other species and other iron-binding proteins were incapable of competing with HRP-bTf even at a 64-fold higher concentration.

**Identification of the bovine transferrin receptor protein.** To identify the protein(s) involved in the specific binding of bovine transferrin to iron-deficient cells or membranes, we employed an affinity isolation procedure that was previously developed for the isolation of meningococcal transferrin and lactoferrin receptor proteins (27). In this procedure, biotinylated bovine transferrin or control protein was mixed with iron-deficient or iron-sufficient membranes to allow for transferrin binding to the membrane receptor. After detergent solubilization of the membrane followed by centrifugation to remove membrane debris, the receptor-transferrin complex was bound to streptavidin-agarose. The resins were washed appropriately before the receptor proteins were eluted from the column in sample buffer and analyzed by SDS-PAGE.

Figure 5 illustrates the results obtained with *P. haemolyt-*

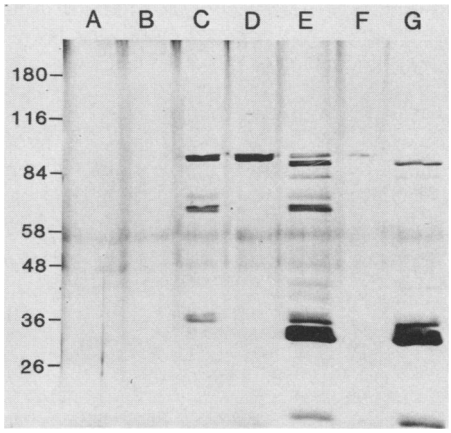


FIG. 5. Identification of the bovine transferrin receptor protein(s) in *P. haemolytica* h44. The receptor proteins were isolated from outer membranes prepared from iron-deficient and iron-sufficient cells as described in Materials and Methods. Samples were analyzed by SDS-PAGE (8 to 10% polyacrylamide), followed by silver staining of the gels. Lanes: A through D and F, samples prepared from affinity isolated preparations; E and G, iron-deficient and iron-sufficient outer membrane preparations, respectively. Except where indicated, affinity isolation utilized the high-salt washing procedure. The source of membrane and the affinity ligand used in the isolation procedure were as follows (lanes): A, iron-deficient outer membranes and biotinylated porcine transferrin; B, iron-deficient outer membranes without added ligand; C, iron-deficient outer membranes and biotinylated bovine transferrin (low-salt wash procedure); D, iron deficient outer membranes and biotinylated bovine transferrin; F, iron-sufficient outer membranes and biotinylated bovine transferrin. Numbers refer to molecular weights (in thousands) of standard proteins.

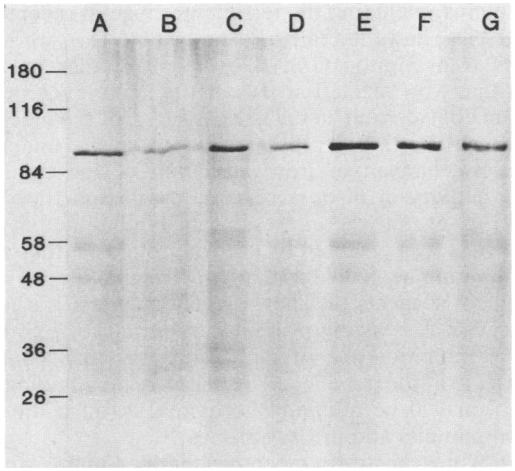


FIG. 6. Comparison of the transferrin receptor protein(s) in different strains of *P. haemolytica* type A1. Samples were prepared from iron-deficient outer membranes by using biotinylated bovine transferrin and the high-salt wash procedure and analyzed by SDS-PAGE as outlined in the legend to Fig. 5. Lanes: A through G, strains h44, h45, h46, PH21, PH24, PH27, and PH45, respectively.

*ica* h44 in the affinity purification procedure. When biotinylated bovine transferrin was used with membranes from iron-deficient cells (lane D), a 100-kDa protein was isolated. Direct SDS-PAGE analysis of iron-deficient (lane E) and iron-sufficient (lane G) outer membrane preparations demonstrated that the isolated protein band was an iron-repressible outer membrane protein. This was further supported by the detectable but low yield of 100-kDa protein isolated when the affinity procedure with biotinylated bovine transferrin was performed with iron-sufficient membranes (lane F). When the affinity isolation procedure using biotinylated bovine transferrin and iron-deficient membranes was performed with less stringent (low-salt wash) conditions, three other membrane proteins of 77, 71, and 37 kDa were detected in addition to the 100-kDa receptor protein. Comparison of lanes E and G demonstrates that the 77- and 71-kDa proteins are iron-repressible outer membrane proteins. When biotinylated porcine-transferrin was substituted for biotinylated bovine transferrin (lane A) or when biotinylated bovine transferrin was omitted from the affinity purification mixture (lane B), the receptor could not be isolated, indicating that the receptor band observed in lane D was specifically isolated by bovine transferrin. It is noteworthy that essentially the same results as illustrated in Fig. 5 were obtained when the affinity purification protocol was performed with total membranes in lieu of outer membranes.

To compare transferrin receptor proteins among different *P. haemolytica* type A1 strains, seven disease isolates were subjected to the affinity isolation procedure. The 100-kDa protein was isolated from all seven strains (Fig. 6). Comparison of the affinity-purified proteins from iron-deficient and iron-sufficient outer membranes (data not shown) demonstrated that the 100-kDa protein was iron repressible in all *P. haemolytica* strains tested.

## DISCUSSION

The requirement for iron in the initiation and maintenance of bacterial infection is well established (5, 32). Vertebrate hosts have evolved specific systems for withholding iron from invading microorganisms while retaining access to this metal (33). In biological fluids such as serum and secretions, extracellular iron is associated with the iron-binding glycoproteins transferrin and lactoferrin (32). The concentration of free iron that remains in equilibrium with these glycoproteins is in the order of $10^{-18}$ M (32) and is far too low to support bacterial growth. Pathogenic bacteria must overcome this iron-restricted environment during the course of natural infection.

Many pathogens obtain iron from otherwise inaccessible sources by excreting high-affinity iron chelators, termed siderophores (21). These systems also involve the synthesis of outer membrane proteins that serve as receptors for the iron-siderophore complexes and aid in their internalization (22). In contrast to the siderophore-mediated mechanism, pathogenic strains of *Neisseria* species (2, 20) and *Haemophilus influenzae* (15) can use transferrin as their sole iron source. The growth of meningococci with transferrin as the sole source of iron depends on surface contact between the meningococcal cell and the transferrin molecule (2). Both iron-deficient cells and isolated membrane preparations bind human transferrin specifically via a cell surface receptor that has been identified and characterized (27, 28).

In the present study, we demonstrate (Fig. 1A and Table 1) that strains of *P. haemolytica* were able to effectively remove iron for growth from 30% saturated bovine transferrin even in the presence of excess EDDA. In contrast, transferrins from other species, including pigs, humans, chickens, and horses, could not serve as the iron source for growth under the same experimental conditions. Iron-deficient whole cells or membranes from these strains specifically bound bovine transferrin only (Fig. 4). These results taken together suggest that the mechanism by which *P. haemolytica* obtains iron from iron-binding proteins is very specific and resembles the receptor-mediated transferrin-iron acquisition mechanism in *N. meningitidis*. Our inability to detect siderophore production in the *P. haemolytica* strains (Table 2) by using a highly sensitive assay (29) suggests that the receptor-mediated pathway may be essential for iron acquisition in vivo.

The observed receptor specificity may partly explain the host specificity of *P. haemolytica* type A1 strains for cattle as well as the low virulence of the organism usually observed in laboratory animals (12). The ability to produce infection in other animals will depend upon the ability to acquire iron for growth in these hosts. In mice, transferrin will not readily be utilized as a source of iron for growth. Previous studies have shown that supply of excess exogenous ferric ammonium citrate enhances *P. haemolytica* infection in mice (1), presumably by overwhelming the host's ability to withhold iron. In addition, an exogenous supply of bovine hemoglobin, which strains of *P. haemolytica* are capable of using (Table 1), results in enhanced *P. haemolytica* infection in mice (6). By analogy with observations of enhancement of meningococcal infection specifically by human transferrin in mice (25), our results (Table 1 and Fig. 1A) predict that only bovine transferrin would enhance *P. haemolytica* infection in other animal species.

The specificity for use of bovine transferrin as a source of iron for growth (Fig. 1) and the detection of a bovine-specific transferrin-binding activity in *P. haemolytica* (Fig. 4) suggested the presence of a functional transferrin receptor in the outer membrane. By using biotinylated bovine transferrin, a protein of 100 kDa was affinity isolated from *P. haemolytica* h44 (Fig. 5). There are several lines of evidence to suggest that the 100-kDa protein is the protein involved in the specific binding to transferrin. First, there was a strong

correlation between the iron regulation of expression of transferrin-binding activity and the 100-kDa protein. In iron-starved cells there was a greatly increased amount of binding activity (Fig. 2) and the 100-kDa protein (Fig. 5, lane E) relative to that observed in iron-replete cells (Fig. 2; Fig. 5, lane G). The relatively rapid expression of the receptor-binding activity (Fig. 3; detectable within 30 min of adding the inducer) correlated with the detection of the 100-kDa band in SDS-PAGE (data not shown). Furthermore, both binding activity (Fig. 2) and the 100-kDa protein (data not shown) were induced by each of the three chelators EDDA, dipyridyl, and desferrioxamine mesylate. Second, the 100-kDa protein was isolated only when biotinylated bovine transferrin was used in the affinity procedure (Fig. 5, lanes C and D) but not when the ligand was omitted from the reaction mixture (Fig. 5, lane B) or when biotinylated porcine transferrin was used as the ligand (Fig. 5, lane A). Conclusive evidence for the role of the 100-kDa protein as the transferrin receptor protein awaits preparation of specific deletion mutants.

The 100-kDa receptor protein isolated from *P. haemolytica* does resemble the upper band of the receptor protein complex described in *Neisseria* species (26, 27) and in *H. influenzae* (24) in several respects. First of all, they are very similar in apparent molecular weight, based on SDS-PAGE analysis. Second, the 100-kDa receptor could not bind to transferrin after SDS-PAGE and electroblotting (data not shown), which was also observed with the upper receptor bands in *Neisseria* species (28) and *H. influenzae* (24). Finally, the upper receptor band was the last to be eluted from the affinity matrix in *Neisseria* species (26) and *H. influenzae* (24), which correlates with the observation that the 100-kDa protein remained tightly bound to the affinity column even under relatively extensive washing procedures (Fig. 5, lane D). One major difference between the purified receptor preparations from *P. haemolytica* and *Neisseria* species or *H. influenzae* is the apparent absence of a lower receptor protein band that is capable of binding transferrin after SDS-PAGE and electroblotting (28). The failure to isolate the lower band, however, may not necessarily indicate that an analogous component is indeed absent in *P. haemolytica*, since the intrinsic properties of this component from *P. haemolytica* may not have favored its isolation by our affinity methods.

In addition to the 100-kDa receptor protein, we were able to isolate two other iron-regulated proteins, a major protein of 71 kDa and a minor protein of 77 kDa (Fig. 5, lane C), by the affinity procedure. These proteins were present in the outer membrane in iron-depleted cells (Fig. 5, lane E) as reported in a previous study (10). In contrast to the previous study, we did not detect the 71-kDa protein in outer membranes from the iron-replete cells in strain h44 (Fig. 5, lane G) or from any of the other serotype 1 strains studied (data not shown). Both the 71- and 77-kDa proteins were eluted under the high-salt wash conditions in which the 100-kDa protein remained bound to the column. The fact that they remained bound to the resin after milder washing conditions may suggest their association in an iron acquisition complex. This hypothesis is consistent with the observation that convalescent sera from calves infected naturally and experimentally reacted with the 100-, 77-, and 71-kDa iron-regulated proteins (10). The precise role of these two proteins and their relationship to the 100-kDa receptor would be better understood through the use of deletion mutants.

Earlier studies in *Escherichia coli* K-12 with rifampin treatment indicated that control of synthesis of iron-repressible proteins, including the ferric enterobactin receptor protein (81 kDa) and the colicin receptor (74 kDa), were at the level of transcription (16). Subsequent studies have confirmed that iron regulation was at the gene level and have provided considerable detailed knowledge of the mechanism of iron regulation. As a preliminary step toward understanding the mechanism of iron regulation of the transferrin-binding activity in *P. haemolytica*, we demonstrate in this study (Fig. 6) that the expression of binding activity in *P. haemolytica* was inhibited by known protein synthesis inhibitors, chloramphenicol and rifampin. The inhibition by chloramphenicol suggests that new protein synthesis rather than modification of existing protein is required for expression, whereas inhibition by rifampin suggests that control of synthesis is at the gene level. A better understanding of the mechanism will be ultimately provided through the use of deletion mutants and mRNA probes.

The bovine transferrin receptor identified in this study has a number of features that make it an ideal candidate for vaccine development. First of all, our study suggests that *P. haemolytica* might be dependent upon the transferrin receptor for iron acquisition in vivo, predicting that loss or drastic alteration of this protein may reduce or eliminate its ability to cause disease. Second, there is evidence for the in vivo production of the 100-kDa protein, which is immunogenic and induces antibody that is cross-reactive with receptors from other strains (10). Third, the function of the receptor dictates in vivo surface accessibility, rendering it available to immunological defense mechanisms. It is possible that some anti-receptor antibody may block transferrin-iron acquisition and thus limit bacterial growth and even potentially reduce the production of the virulence factor bovine leukotoxin.

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