Cloning and Expression of a Transferrin-Binding Protein from *Actinobacillus pleuropneumoniae*†

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An expression library was constructed from *Actinobacillus pleuropneumoniae* serotype 7. *Escherichia coli* transformants expressing recombinant proteins were identified by immunoscreening with porcine convalescent serum. One transformant expressing a 60-kDa protein (60K protein) in aggregated form was identified. Serum raised against the recombinant protein recognized a polypeptide with an indistinguishable electrophoretic mobility in the *A. pleuopneumoniae* wild type after iron-restricted growth only. The recombinant protein bound transferrin after blotting onto nitrocellulose. Using a competitive enzyme-linked immunosorbent assay (ELISA), the specificity of this binding for the amino-terminal half of iron-saturated porcine transferrin was established. Also, the 60K wild-type protein bound hemin as assessed by hemin-agarose chromatography. Hemin could inhibit transferrin binding of the recombinant protein in the competitive ELISA, whereas hemoglobin and synthetic iron chelators failed to do so. Southern blot analysis of several other *A. pleuropneumoniae* strains indicated that highly homologous sequence is present in eight of eight isolates of serotype 7 and in some isolates of serotypes 2, 3, and 4.

The ability of microorganisms to bind and utilize transferrin as a sole iron source as well as the correlation between virulence and the ability to scavenge iron from the host has long been established (2, 3, 11, 29). The species specificity of bacterial transferrin-binding ability in vivo was shown by Holbein (12, 13), who could enhance a *Neisseria meningitidis* infection in mice by the injection of human transferrin. It was also observed that, despite their lack of siderophore production (20, 30), *Neisseria* spp. expressed new outer membrane proteins in response to iron limitation (19). Also, Simonson et al. (25) showed that the ability of *N. meningitidis* to take up iron from transferrin is dependent on its previous cultivation under iron-limiting conditions. Subsequently, Schryvers and Morris (23, 24) demonstrated the binding of transferrin and lactoferrin to iron-regulated outer membrane proteins in this species. In addition, the specificity of bacterial transferrin binding for the proteins of the natural host has been confirmed for other species by an in vitro binding assay (7, 18, 21).

Actinobacillus pleuropneumoniae is a porcine respiratory tract pathogen which causes acute fibrinous pneumonia or chronic lung lesions in infected animals. *A. pleuropneumoniae*, under iron-restricted growth conditions, can use porcine transferrin, hemoglobin, and various porphyrins as its sole iron source, but it cannot utilize bovine or human transferrin (5, 7, 18). Iron limitation induces the expression of several new outer membrane proteins. These enable the cells to bind hemin and Congo red in a competitive fashion (5), and in addition, two of the iron-regulated proteins have been shown to bind transferrin in an in vitro binding assay (7).

The molecular mechanisms of the porphyrin and transferrin binding, as well as the metabolic pathway(s) involved in utilizing the iron, have not yet been elucidated. To investi-

gate these questions, we cloned a transferrin- and hemin-binding protein from *A. pleuropneumoniae* serotype 7. We describe the expression of the antigenically active protein in *Escherichia coli* and show that the recombinant protein has binding characteristics indistinguishable from those described for *A. pleuropneumoniae* cells.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *A. pleuropneumoniae* serotype 7 strain AP205 is a Nebraska clinical isolate kindly provided by M. L. Chepok, Modern Veterinary Products, Omaha, Nebr. One *A. pleuropneumoniae* serotype 2 strain and the serotype 4 strain were obtained from the American Type Culture Collection (ATCC 27089 and ATCC 33378). The other *A. pleuropneumoniae* strains are field isolates from herds in Saskatchewan. *E. coli* HB101 (16) and JM105 (16) were used in all transformation experiments. Plasmids pGH432 and pGH433 are expression vectors containing a *tac* promoter, a translational start site with restriction enzyme sites allowing ligation in all three reading frames, and stop codons in all reading frames (1). *A. pleuropneumoniae* strains were grown on PPLO medium (Difco Laboratories, Detroit, Mich.) supplemented with IsoVitaleX (1% [vol/vol]; BBL Microbiology Systems, Becton Dickinson & Co., Cockeysville, Md.). Iron restriction was obtained by adding 2,2'-dipyridyl to a final concentration of 100 µM. Heat stress was induced by transferring cultures to 45°C for 2 h. Ethanol stress was exerted by the addition of 10% (vol/vol, final concentration) of absolute ethanol to cultures in the mid-log phase. Oxidative stress was induced by the addition of 1% (vol/vol, final concentration) of 30% H_2O_2 to the cultures. *E. coli* transformants were grown in Luria medium (16) supplemented with ampicillin (100 mg/liter) and induced to form aggregates by the addition of isopropyl-thiogalactoside (IPTG, 1 mM final concentration). To detect Congo red or hemin binding, we supplemented the ampicil-

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lin-containing Luria agar with 1 to 10 μM IPTG and 0.003% Congo red or 0.02% hemin.

Preparation and analysis of culture supernatants, outer membranes, and protein aggregates. Culture supernatants were obtained by pelleting *A. pleuropneumoniae* cells grown to the mid-log phase at 4,000 × *g* for 20 min and further separated by ultracentrifugation at 100,000 × *g* for 30 min. The supernatants after low- or high-speed centrifugation were concentrated by precipitation with 2 volumes of absolute ethanol and kept at −20°C for 1 h. Precipitates were recovered by centrifugation and resuspended in water. Total membranes were prepared by French press treatment and subsequent centrifugation at 100,000 × *g* (9). Outer membranes were prepared by sucrose gradient centrifugation with a two-step gradient (8) and by Sarkosyl solubilization (5). The integrity of bacterial cells at the mid-log phase of growth was ascertained by β-galactosidase assay (16) of cultures and culture supernatants obtained by low-speed centrifugation.

For the preparation of protein aggregates, broth cultures (50 ml) in the mid-log phase were induced by the addition of 1 mM IPTG (final concentration). After 2 h of vigorous shaking, cells were harvested by centrifugation, resuspended in 2 ml of 25% sucrose–50 mM Tris-HCl buffer (pH 8), and frozen at −70°C. Lysis was achieved by the addition of 5 μg of lysozyme in 250 mM Tris-HCl buffer (pH 8), 5 min of incubation on ice, addition of 10 ml of detergent mix (5 parts of 20 mM Tris-HCl buffer [pH 7.4]–300 mM NaCl–2% deoxycholic acid–2% Nonidet P-40 and 4 parts of 100 mM Tris-HCl buffer [pH 8]–50 mM EDTA–2% Triton X-100), and sonication. Protein aggregates were harvested by centrifugation for 30 min at 15,000 × *g*. Aggregate protein was resuspended in H$_2$O to a concentration of 5 to 10 mg/ml and dissolved by the addition of an equal volume of 7 M guanidine hydrochloride.

Proteins were analyzed by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (14). The protein concentration was estimated by comparing the intensity of the Coomassie blue-stained bands to a bovine serum albumin standard (Pierce Chemical Co., Rockford, Ill.). Electrophoretic transfer onto nitrocellulose membranes was performed essentially as described by Towbin et al. (27). Nonspecific binding was blocked by incubation in 0.5% gelatin in washing buffer (150 mM NaCl, 30 mM Tris-HCl [pH 8], 0.05% Triton X-100). Antibody and alkaline phosphatase conjugate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) were added in washing buffer and each was incubated for 1 h at room temperature. Blots were developed with a substrate containing 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue tetrazolium (ImmunoSelect; Bethesda Research Laboratories, Inc., Gaithersburg, Md.) in 100 mM Tris/HCl buffer (pH 9.5), 50 mM NaCl, 5 mM MgCl$_2$.

Preparation of antisera. Convalescent serum was obtained by exposing pigs in an aerosol chamber (22) to 1 × 10^{-5} 50% lethal dose (LD$_{50}$) (1 × 10^3 CFU/ml of aerosolized bacterial suspension) of *A. pleuropneumoniae* serotype 7 and challenging them 2 weeks later with 2 LD$_{50}$. Serum against the recombinant protein was raised in mice by intraperitoneal injection of 30 μg of dissolved aggregate in complete Freund's adjuvant and a subcutaneous boost with 30 μg of protein in incomplete Freund's adjuvant.

Iron compounds. Transferrins from different species were obtained commercially (porcine transferrin from The Binding Site, Birmingham, United Kingdom; human and bovine transferrin from Sigma Chemical Co., St. Louis, Mo.).

Porcine transferrin was iron depleted as described by Mazurier and Spik (17). The resulting porcine apotransferrin as well as the commercially obtained bovine and human apotransferrins were iron saturated as described by Herrington and Sparling (11). The amino-terminal fragment of porcine transferrin was prepared by the method of Lineback-Zins and Brew (15).

Transferrin and hemin binding assays. To assess the possible transferrin-binding ability of recombinant proteins, we performed a Western blot-like (immunoblot) transferrin-binding assay essentially as described by Morton and Williams (18). Briefly, the protein was resuspended in a nonreducing sample buffer, and during the entire procedure the temperature was kept below 37°C. Blots were developed with biotinylated transferrin (Biotin-XX-NHS ester Labeling Kit; Clontech Laboratories, Palo Alto, Calif.) coupled to streptavidin-phosphatase and purified by gel filtration with a G-100 column.

To detect a hemin-binding protein(s) of *A. pleuropneumoniae*, sucrose gradient-purified outer membranes from cells grown under iron-restricted conditions were suspended in TM buffer (50 mM Tris [pH 8.0], 5 mM MgCl$_2$, 0.1% [vol/vol] Triton X-100), incubated for 2 h at room temperature, and centrifuged at 100,000 × *g*. The supernatant was loaded onto a hemin-agarose column as described by Hanson and Hansen (10). The hemin-binding protein was eluted with TM buffer containing 0.5, 1, and 2 M NaCl or 2 M guanidine hydrochloride. Hemin binding was further assessed by using hemin as the competitive agent in the enzyme-linked immunosorbent assay (ELISA) described below. To exclude an effect of nonspecific hemin binding to transferrin, an excess of bovine transferrin (1 mg/ml) was included in the incubation.

To determine species specificity of transferrin binding, we developed a competitive ELISA. ELISA plates (Immulon 2; Dynatech Laboratories, Inc., Alexandria, Va.) were coated with 100 μl of porcine transferrin at a concentration of 100 μg/ml in carbonate buffer at 4°C overnight. All subsequent steps were performed at room temperature. Plates were blocked with 0.5% gelatin in washing buffer. Dissolved protein at a concentration of 2 μg/ml in washing buffer containing 0.5% gelatin was incubated for 1 h with an equal volume of serial twofold dilutions of porcine, bovine, and human transferrin as well as hemin and other possible inhibitors in washing buffer. Subsequently, 200 μl of this solution was added to the coated and washed wells and incubated for 1 h. The assay was developed with the mouse serum raised against the recombinant 60-kDa protein (60K protein), an alkaline phosphatase-labeled conjugate, and *p*-nitrophenyl phosphate in 1 M diethanolamine (pH 9.5)–5 mM MgCl$_2$ as the substrate. The plates were read at 405 nm, and the inhibition values state the percentage by which a certain concentration of competitor in the preincubation step inhibits the reaction between the recombinant 60K protein and the coating transferrin.

Cloning of the gene encoding a transferrin-binding protein. All restriction enzyme digests were done in T4 DNA polymerase buffer (16) containing 1 mM dithiothreitol and 3 mM spermidine. Genomic DNA was prepared as previously described (26) and partially digested with the restriction endonuclease *Sau*3AI. Fragments of 1,500 to 2,500 bp were isolated by sucrose density gradient centrifugation (16) and ligated into pGH432 and pGH433. *E. coli* HB101 transformants were replica plated onto nitrocellulose discs (Bio-Rad Laboratories, Richmond, Calif.) and induced for 2 h on plates containing 1 mM IPTG. Transformants expressing *A.*
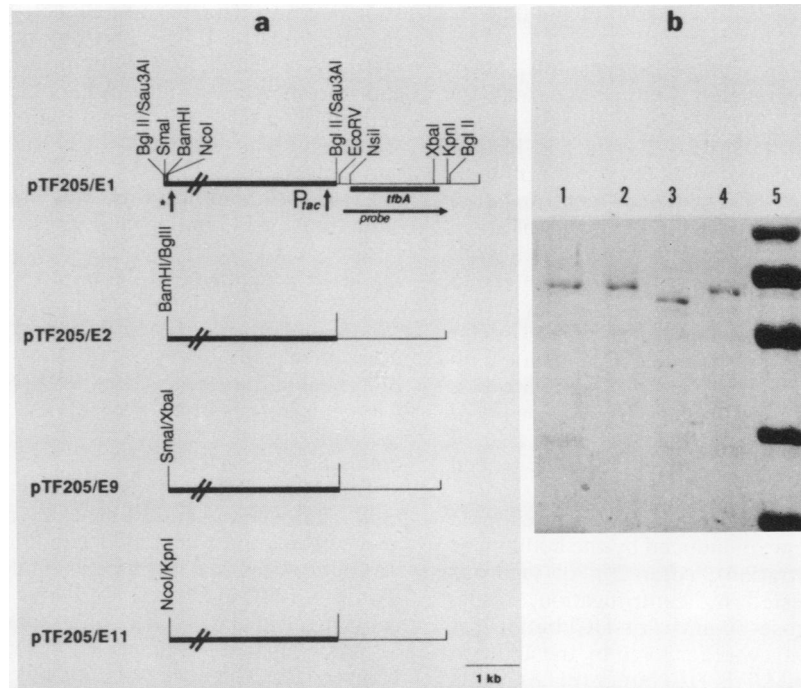
FIG. 1. Physical map and translational activity of plasmid pTF205/E1 and its deletion derivatives pTF205/E2, pTF205/E9, and pTF205/E11. (a) The thick line represents DNA of the cloning vehicle (pGH433), P_{tac} indicates the location of the *tac* promoter, and the asterisk indicates stop codons in all three reading frames. The horizontal arrow indicates the location and direction of transcription of the encoding gene designated *tfbA*. The *Eco*RV-*Bgl*II fragment was used as a probe. (b) SDS-polyacrylamide gel of the IPTG-induced aggregate proteins produced by pTF205/E1 (lane 1), pTF205/E2 (lane 2), pTF205/E9 (lane 3), and pTF205/E11 (lane 4); lane 5 depicts a molecular weight standard containing phosphorylase *b* (97,400), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), and soybean trypsin inhibitor (21,500).

pleuropneumoniae antigens were detected by immunoblotting with pig convalescent serum, conjugate, and substrate as described above. Positive transformants were replated, induced, and analyzed by Western blot, using as a control a whole-cell lysate of *A. pleuropneumoniae* grown under iron-limiting conditions. The identity of the encoding DNA was confirmed by Southern blot analysis as described by Maniatis et al. (16). The washing conditions were $0.1 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% SDS and 68°C.

RESULTS

Identification of the 60K protein-expressing *E. coli* transformant. Of approximately 6,000 transformants screened by the immunoblot method, 22 reacted with the convalescent serum and showed an immunoreactive band in the Western blot analysis. One transformant expressed a protein with the same electrophoretic mobility as an *A. pleuropneumoniae* polypeptide present only under iron-limiting growth conditions. Plasmid was prepared from this transformant and designated pTF205/E1 (Fig. 1a). Three deletion derivatives were constructed and designated as pTF205/E2, pTF205/E9, and pTF205/E11 (Fig. 1a). *E. coli* HB101 transformed with any of these plasmids, in the absence of IPTG as well as after induction with low concentrations of IPTG (1 to 10 μM), bound both Congo red and hemin. Upon induction with 1 mM IPTG, the *E. coli* transformants produced inclusion bodies consisting of aggregated 60K protein (Fig. 1b), and the protein isolated from pTF205/E9 transformants appeared to be smaller by approximately 5 kDa. The pTF205/E1

transformants, in addition, seemed to produce a 30K protein absent in the other transformants.

Iron-regulated expression of *A. pleuropneumoniae* 60K protein and its ability to bind transferrin and hemin. In a Western blot, the serum raised against the recombinant 60K protein detected a single polypeptide in whole-cell lysates of *A. pleuropneumoniae* grown under iron-restricted conditions. The protein was not expressed under conditions of heat, ethanol, or oxidative stress (Fig. 2a). After transfer onto nitrocellulose, a 60K protein in whole-cell lysates from *A. pleuropneumoniae* grown under iron-restricted conditions as well as the recombinant protein reacted with biotinylated porcine transferrin (Fig. 2b). In addition, a 30K, a 75K, and a 100K protein were stained by biotinylated transferrin in *A. pleuropneumoniae* grown under iron-restricted conditions. The 75K protein was also present in *A. pleuropneumoniae* grown under standard conditions, and a 30K protein was present both in *A. pleuropneumoniae* grown under standard conditions and in *E. coli* (Fig. 2b). The hemin-agarose column retained a 60K protein from *A. pleuropneumoniae* grown under iron-restricted conditions. The protein was eluted with 2 M guanidine hydrochloride, and it reacted with serum raised against the recombinant 60K protein (Fig. 2c).

Localization of 60K protein in *A. pleuropneumoniae* cultures. Whole-cell lysates, total membranes, outer membranes as prepared by sucrose gradient centrifugation, and low-speed culture supernatants contained the 60K protein but no detectable β-galactosidase activity. Outer membranes prepared by sarcosyl solubilization did not contain the 60K protein (Fig. 3a). Pellets from the high-speed centrifugation
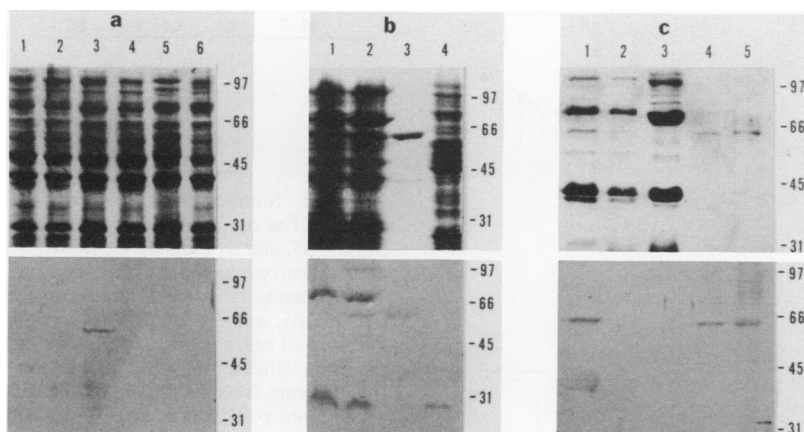
FIG. 2. *A. pleuropneumoniae* 60K protein is induced by iron restriction and binds porcine transferrin and hemin. (a) Expression of the 60K protein by *A. pleuropneumoniae* AP205 upon induction with different stimuli. Whole-cell lysates stained with Coomassie blue after SDS-PAGE (top) and corresponding Western blot using a serum raised against recombinant 60K protein (bottom); lanes: 1, common start culture; 2, control culture; 3, iron-limiting growth conditions; 4, ethanol stress; 5, peroxide stress; 6, heat shock. (b) Transferrin binding of the *A. pleuropneumoniae* 60K protein. Coomassie blue-stained gel (top) and the same preparations on a nitrocellulose membrane incubated with alkaline phosphatase-labeled porcine transferrin (bottom); lanes: 1, whole-cell lysate of *A. pleuropneumoniae* AP205 control cells; 2, cells grown under iron-limiting growth conditions; 3, aggregate protein prepared from pTF205/E1 transformants; 4, *E. coli* HB101 whole-cell lysate. (c) Hemin binding of the *A. pleuropneumoniae* 60K protein. Coomassie blue-stained gel (top) and the corresponding Western blot using the serum raised against the recombinant 60K protein (bottom); lanes: 1, sucrose gradient-purified outer membranes; 2, Triton X-100-treated membranes; 3, outer membranes prepared by Sarkosyl solubilization; 4, hemin-agarose eluent at 2 M guanidine hydrochloride; 5, aggregate protein from pTF205/E1 transformants. The positions and molecular weights ($\times 10^3$) of marker proteins (phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase) are indicated to the right of each gel.

of culture supernatants contained the 60K protein, whereas it was not detected in the supernatants (Fig. 3b).

Functional characterization of the recombinant 60K protein. Aggregate protein resuspended in nonreducing sample buffer and subjected to SDS-PAGE was found to bind alkaline phosphatase-labeled porcine transferrin (Fig. 2b). The species specificity of this binding was demonstrated in a competitive ELISA. Thus, the binding of dissolved aggregate protein to iron-saturated porcine transferrin could be inhibited completely by iron-saturated porcine transferrin and was 50% inhibited by 0.2 μM porcine transferrin (Fig. 4). In contrast, approximately 3.0 μM of porcine apotransferrin was necessary to produce 50% inhibition (Fig. 4). Using human iron-saturated transferrin, only 40% inhibition could be obtained even with a concentration 60 times higher than the 50% inhibitory concentration of porcine transferrin, and no inhibition was seen with bovine transferrin. In addition, 6 μM hemin could inhibit transferrin binding of the 60K
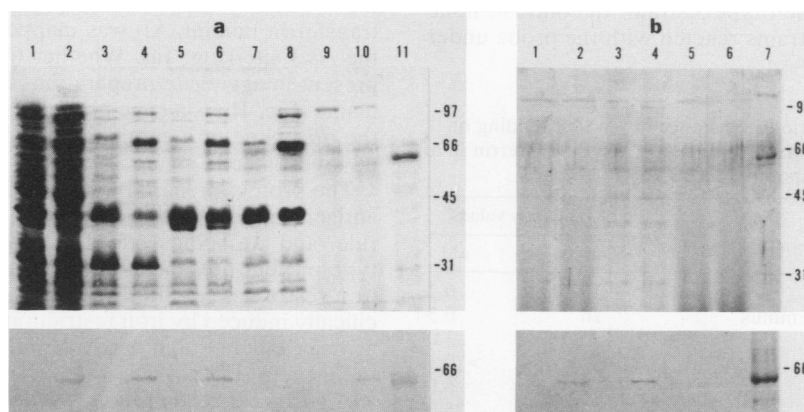

FIG. 3. Localization of the 60K protein in *A. pleuropneumoniae* AP205 showing a gel stained with Coomassie blue (top) and the corresponding Western blot using a serum raised against the recombinant 60K protein (bottom). (a) Whole-cell lysates from *A. pleuropneumoniae* grown under standard (lane 1) and iron-restricted (lane 2) conditions, the corresponding total membranes (lanes 3 and 4), the outer membranes prepared by sucrose gradient centrifugation (lanes 5 and 6) or Sarkosyl solubilization (lanes 7 and 8), culture supernatants prepared by low-speed centrifugation (lanes 9 and 10), and aggregate protein prepared from pTF205/E1 transformants (lane 11). (b) Supernatants after low-speed centrifugation from an *A. pleuropneumoniae* culture grown under standard (lane 1) and iron-restricted (lane 2) conditions, corresponding pellets (lanes 3 and 4) and supernatants (lanes 5 and 6) obtained by ultracentrifugation, and aggregate protein prepared from pTF205/E1 transformants (lane 7). The positions and molecular weights ($\times 10^3$) of marker proteins (phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase) are indicated to the right of each gel.
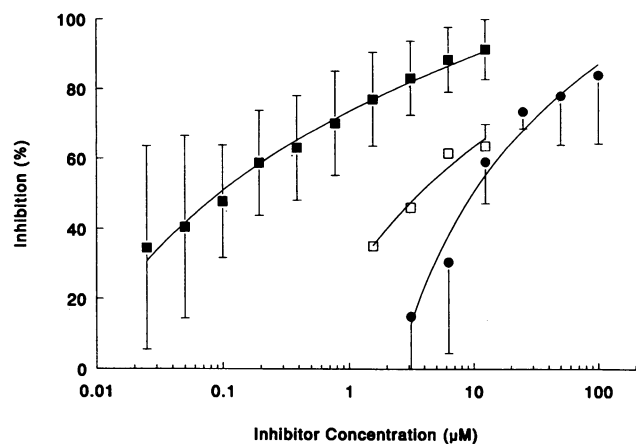
FIG. 4. Inhibition of transferrin binding by the recombinant 60K protein in the competitive ELISA. The inhibitory compounds depicted are iron-saturated porcine transferrin (■), porcine apotransferrin (□), and hemin (●). The points are the weighted means from two to five experiments done in duplicate, with the vertical bars depicting the standard deviations. Different aggregate preparations, different sera, and different transferrin preparations were used in the individual experiments.

protein by 50%, and the inhibitory effect was unaltered in the presence of 1 mg of bovine transferrin per ml (Fig. 4). Congo red also was inhibitory, whereas porcine hemoglobin, ethylenediamine-diorthohydroxyphenyl-acetate (EDDA), 2,2'-dipyridyl, and ferric citrate did not have an inhibitory effect in this assay (Table 1).

Distribution of the 60K protein-encoding gene among different serotypes of A. pleuropneumoniae. Chromosomal DNA was prepared from 27 isolates of *A. pleuropneumoniae* belonging to six different serotypes, digested with the restriction endonucleases *BglII* and *EcoRV*, and separated on an agarose gel. A Southern blot analysis with the *EcoRV-BglII* fragment of pTF205/E1 as a probe showed that a fragment identical in size to the one in pTF205/E1 was present in all *A. pleuropneumoniae* serotype 2, 4, and 7 strains as well as in one serotype 3 strain. In contrast, none of the serotype 1 and 5 strains reacted with the probe under

TABLE 1. Ability of various substances to inhibit binding of recombinant 60K protein to iron-saturated porcine transferrin in a competitive ELISA

Competitive substances[a]	50% inhibition values[b]	
	μg/ml	μM
Porcine transferrin	15[c]	0.2
Porcine transferrin, NH₂ terminus	20	0.5
Porcine apotransferrin	250	3.0
Human transferrin	>1,000[d]	>12.5
Bovine hemin	4	6.0
Congo red	25	35.0

[a] Also tested and completely noninhibitory were bovine transferrin (12.5 μM), porcine hemoglobin (14 μM), EDDA (100 μM, iron saturated), 2,2'-dipyridyl (100 μM, iron saturated), and ferric citrate (10 mM).

[b] Inhibition values state the concentration of competitor in the preincubation step necessary to inhibit by 50% the reaction between recombinant 60K protein and coating transferrin.

[c] The value varied among experiments between 12.5 and 100 μg/ml (Fig. 4); however, the relative difference in inhibitory activity between the various substances was constant.

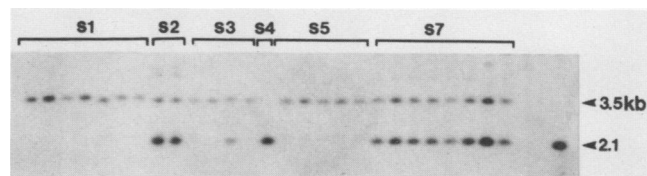[d] This concentration inhibited binding by approximately 40%.

FIG. 5. Southern blot analysis of different *A. pleuropneumoniae* isolates. The origin of the probe used is indicated in Fig. 1; S1, S2, S3, S4, S5, and S7 indicate the positions of chromosomal DNA from the *A. pleuropneumoniae* serotypes 1, 2, 3, 4, 5, and 7, respectively. The following lanes contain control DNA from *E. coli* HB101, *E. coli* JM105, and *E. coli* HB101 transformed with pTF205/E1. The DNA in all cases is cut with *EcoRV* and *BglII*. The arrowheads to the right indicate the positions and sizes of the hybridizing restriction enzyme fragments. The 2.1-kb fragments hybridized with the probe from pTF205/E1. The 3.5-kb fragments hybridized to an *A. pleuropneumoniae* cytolysin probe (1) used as a control to show the integrity of the restricted DNA.

high-stringency washing conditions (Fig. 5). To show the integrity of the DNA from the nonreactive strains, we added a 3.5-kb *BglII* fragment from an *A. pleuropneumoniae* cytolysin-containing bacteriophage clone (1) as a control probe. Also, under low-stringency washing conditions (3× SSC, 55°C), weak hybridization of pTF205/E1-derived probe to DNA from *A. pleuropneumoniae* serotype 1 and 5 isolates was observed (data not shown).

DISCUSSION

In the present report, we described the cloning and expression in *E. coli* of a functionally active transferrin- and hemin-binding protein from *A. pleuropneumoniae* serotype 7. *E. coli* transformants carrying the recombinant plasmid could be induced with IPTG to produce inclusion bodies consisting of the aggregated polypeptide. The truncated protein produced by pTF205/E9 transformants (Fig. 1b) indicates that the 3' end of the encoding gene is located approximately 150 nucleotides downstream from the *XbaI* site. Therefore, based on the total size of the polypeptide, the 5' end of the encoding gene (designated *tfbA* [for transferrin binding A]) was mapped to be approximately at the *NsiI* site (Fig. 1a). Whether the additional 30K protein present in aggregate preparations from pTF205/E1 transformants (Fig. 1b) is a gene product encoded downstream from the *tfbA* gene or a contaminating *E. coli* protein remains to be investigated.

The protein prepared from pTF205/E2-transformants was antigenically active, when dissolved in guanidine hydrochloride, and antibodies raised against it detected a 60K wild-type protein present in *A. pleuropneumoniae* grown under iron-restricted conditions. Expression of the protein is specifically induced by iron restriction since other stresses such as heat, ethanol, and peroxide did not induce production of the protein (Fig. 2a).

A 60K wild-type protein was shown to bind transferrin in a Western blot-like assay (Fig. 2b). Three other proteins of approximately 30, 75, and 100 kDa molecular mass also bound transferrin in this assay (Fig. 2b). The 30K protein was not located in the membrane (data not shown). The 75K protein was present in the bacterial lysates independent of the growth conditions, and it was found to bind streptavidin-phosphatase alone (data not shown). The 100K protein was found only in cells grown under iron-restricted conditions, and it appeared to be present in substantial amounts in the

outer membrane (Fig. 3a). Gonzalez et al. (7) recently described the transferrin-binding ability of 56K and 105K outer membrane proteins of *A. pleuropneumoniae*. We suggest that the cloned 60K protein is related to the 56K protein identified by these researchers. Our results using hemin-agarose affinity chromatography and subsequent Western blotting indicate that the 60K protein additionally has the ability to bind hemin (Fig. 2c). Our finding that the protein could not be eluted from the column by using 2 M NaCl as described for the hemin-binding protein from *Haemophilus influenzae* (10) suggests a relatively high affinity binding.

In *A. pleuropneumoniae*, the 60K protein was found to be present in outer membranes prepared by sucrose gradient centrifugation but absent in outer membranes prepared by Sarkosyl solubilization (Fig. 3a). This indicates that it is different from the iron-regulated outer membrane proteins previously described by Deneer and Potter (5). However, this finding is consistent with the results from Banerjee-Bhatnagar and Frasch (4), who have shown that a 70K transferrin-binding protein from *N. meningitidis* can be separated from outer membranes by detergent treatment. Our results are also consistent with the findings of Hanson and Hansen (10), who showed that the hemin-binding protein from *H. influenzae* can be removed from outer membranes by treatment with Triton X-100. The presence of the 60K protein in the supernatant of *A. pleuropneumoniae* serotype 7 cultures in the mid-log phase could be due to either cell lysis or secretion or the formation of membrane vesicles which have been observed to be produced by *A. pleuropneumoniae* isolates (unpublished data) as well as by other gram-negative pathogens (6). To investigate this question, we assayed cell cultures and supernatants for β-galactosidase activity. The absence of detectable amounts of enzyme activity in culture supernatants suggested that cell lysis had not occurred to a significant degree. Also, low-speed supernatants were further separated by ultracentrifugation. The presence of detectable amounts of the 60K protein in the ultracentrifugation pellet and its absence in the supernatant (Fig. 3b) indicate that the presence of the 60K protein in culture supernatants is due to the formation of membrane vesicles.

After SDS-PAGE under nonreducing conditions, the recombinant protein was found to bind biotinylated transferrin in a Western blot-like assay (Fig. 2a). To rule out unspecific transferrin binding by the recombinant protein, we developed a competitive ELISA. This assay showed that a 15-fold-higher amount of porcine apotransferrin than iron-saturated porcine transferrin was necessary to inhibit binding to the coating iron-saturated porcine transferrin by 50% (Fig. 4). Fifty percent inhibition could not be achieved with human or bovine transferrin with concentrations 60 times greater than those effective with iron-saturated porcine transferrin. The inability to bind human and bovine transferrin is consistent with the previously reported inability of *A. pleuropneumoniae* wild-type strains to use these transferrins as sources of iron (7). Therefore, this result indicates that the transferrin binding of the recombinant protein is due to the same mechanism used by the native polypeptide. In addition, the stronger inhibition of 60K protein transferrin binding by iron-saturated transferrin demonstrates that the conformational change of transferrin due to iron saturation influences its affinity for the 60K protein. This finding suggests that in vivo, *A. pleuropneumoniae* is able to efficiently distinguish between iron-saturated and iron-depleted transferrin. This is in contrast to *N. meningitidis*, which has

been found to be unable to distinguish between iron-saturated and iron-depleted transferrin (28).

Of further interest is the ability of the 60K protein to bind the amino terminus of the transferrin molecule. This, as well as the preference for iron-saturated transferrin, indicates that binding does not occur in the hinge region of the molecule but in an area of the amino-terminal loop which is exposed or conformationally altered in the iron-saturated state.

The inhibitory activity of hemin in this assay was consistent with the binding of the 60K wild-type protein to hemin-agarose as well as the Congo red and hemin binding phenotype of *E. coli*(pTF205/E1) transformants. Also, to exclude nonspecific interaction between the coating transferrin and the hemin, the competitive ELISA with hemin was done both with and without 1 mg of bovine transferrin per ml. No difference in the inhibitory effect of hemin was noticed, thus suggesting that the inhibition is caused by hemin binding of the 60K protein. However, whether this inhibition is due to competitive or allosteric inhibition remains to be investigated. Since *A. pleuropneumoniae* is able to grow on both hemin and transferrin as the only iron source, the possibility of one common mechanism used to obtain iron from both substances poses an interesting area for future research.

Based on the mapped location of the *tfbA* gene, the *EcoRV*-*BglII* fragment was chosen as the DNA probe to confirm the identity of the cloned DNA and its distribution in other *A. pleuropneumoniae* isolates. The Southern blot analysis demonstrates that the *tfbA* gene is conserved among the serotype 2, 4, and 7 strains investigated. The absence of hybridization under high-stringency conditions to DNA from *A. pleuropneumoniae* serotype 1 and 5 isolates suggests a divergence of the *tfbA* gene among different serotypes. This has subsequently been confirmed by Western blot analysis and by cloning of the *tfbA* gene from an *A. pleuropneumoniae* serotype 1 isolate (6a).

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