INFECTION AND IMMUNITY, Oct. 1995, p. 3846–3850 Vol. 63, No. 10
0019-9567/95/$04.00+0

Mapping of Functional Regions on the Transferrin-Binding Protein (TfbA) of *Actinobacillus pleuropneumoniae*

KATRIN STRUTZBERG,[1] LARS VON OLLESCHIK,[2] BURKART FRANZ,[1] CARON PYNE,[3] M. ALEXANDER SCHMIDT,[2] AND GERALD-F. GERLACH[1]*

Institut für Mikrobiologie und Tierseuchen, Tierärztliche Hochschule Hannover, 30173 Hannover,[1] and Institut für Infektiologie, Zentrum für Molekularbiologie der Entzündung, 48129 Münster,[2] Germany, and Veterinary Infectious Disease Organization, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 0W0[3]

Received 3 April 1995/Returned for modification 19 June 1995/Accepted 24 July 1995

***Actinobacillus pleuropneumoniae* can use porcine transferrin as the sole source of iron. Two proteins with molecular masses of approximately 60 kDa (TfbA) and 110 kDa have been shown to specifically bind porcine transferrin; from the TfbA protein, three isoforms from *A. pleuropneumoniae* serotypes 1, 5, and 7 have been identified and characterized by nucleotide sequence analysis. Here we defined the transferrin-binding region(s) of the TfbA protein of *A. pleuropneumoniae* serotype 7 by Tn*phoA* mutagenesis, random mutagenesis, and peptide spot synthesis. The amino-terminal half of the TfbA molecule, which has only 36% amino acid sequence identity among the three isoforms, was shown to be responsible for transferrin binding by Tn*phoA* mutagenesis. This result was confirmed by analysis of six random mutants with decreased transferrin binding affinity. The subsequent analysis of overlapping 16-mer peptides comprising the amino-terminal half of the TfbA molecule revealed three domains of 13 or 14 amino acids in length with transferrin-binding activity. They overlapped, or were very close to, point mutations decreasing transferrin-binding ability. The first and third domains were unique to the TfbA protein of *A. pleuropneumoniae* serotype 7. In contrast, the sequence of the second domain was present in almost identical forms (12 of 14 residues) in the TfbA proteins of *A. pleuropneumoniae* serotypes 1 and 5; in addition, a sequence consisting of functionally homologous amino acids was present in the otherwise completely distinct small transferrin-binding proteins of *Neisseria gonorrhoeae* (TbpB), *N. meningitidis* (Tbp2), and *Haemophilus influenzae* (Tbp2).**

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia, a highly contagious disease encountered worldwide (32). Therapeutic measures are difficult because of the rapid onset of disease and an increasing occurrence of isolates with multiple-antibiotic resistance phenotypes. Therefore, different active immunization strategies have been attempted with varied success (5, 16, 17, 29). The major obstacle to establishment of a successful vaccination strategy is the occurrence of 12 serotypes of *A. pleuropneumoniae*; thus, immunization with a bacterin from one serotype does not induce protection against infection with other serotypes (17). This problem also occurred when recombinant antigens were used for vaccination (29). One of the protective recombinant antigens evaluated is the TfbA protein from *A. pleuropneumoniae* serotype 7, an antigen of which three different isoforms have been identified in the various serotypes (2, 10). It was observed that convalescent-phase serum as well as serum from immunized pigs mainly recognized an epitope localized on the carboxy-terminal fifth of the molecule and that deletion of this part of the molecule did not influence transferrin binding (9). Since transferrin binding is a rather specific function (12, 28), it was hypothesized that the binding region(s) might be conserved among the different TfbA isoforms. Thus, antibodies raised specifically against a transferrin-binding region might be able to induce a cross-protective immune response. Alternatively, peptides comprising either the entire transferrin-binding or at least part of this region might be able to compete with the bacterial protein for binding to transferrin and thus interfere with the essential iron uptake. The precise localization of the transferrin-binding region(s) of the TfbA protein would subsequently allow a comparison with other known transferrin-binding proteins; thus, it could be elucidated whether in principle the same sequence is responsible for transferrin binding of functionally similar proteins from different organisms such as *Neisseria* spp.

In order to localize functional regions on a molecule, a variety of methods have been used successfully. Best results are to be expected from a combination of a genetic approach (in vivo and in vitro mutagenesis) and a subsequent peptide synthesis approach (11). The latter has become considerably easier to handle by a new method allowing the direct coupling of peptides onto a nitrocellulose membrane (spot synthesis [8]). In this study, we have localized the transferrin-binding region of the TfbA protein from *A. pleuropneumoniae* serotype 7 by using transposon and random mutagenesis (6, 23) as well as a peptide spot synthesis approach (8).

MATERIALS AND METHODS

Bacterial strains, media, plasmids, iron compounds, and antisera. *Escherichia coli* HB101 [*hsdS20*($r_B^-m_B^-$) *supE44 recA13*] transformed with the recombinant plasmid pTF205/E2 (9) was grown on Luria medium containing ampicillin (100 μg/ml). Plasmid pTF205/E2 contains the *A. pleuropneumoniae* serotype 7-derived *tfbA* gene ligated into the *Bgl*II site of pGH433 (a *lac* repressor and β-lactamase-encoding pBR derivative with a *tac* promoter followed by a unique *Bgl*II site and stop codons in all three reading frames). This construct results in translation of a complete TfbA protein (including signal peptide) and partial membrane transport, thus allowing the detection of functional TfbA in a colony blot.

Bovine apotransferrin was obtained commercially (Sigma Chemical Co., St. Louis, Mo.). Porcine transferrin was prepared by ammonium sulfate precipitation and subsequent ion-exchange chromatography as described by Niven et al. (26) and iron depleted as described by Mazurier and Spik (24). Porcine and

* Corresponding author. Mailing address: Institut für Mikrobiologie und Tierseuchen, Tierärztliche Hochschule Hannover, Bischofsholer Damm 15, D-30173 Hannover, Germany. Phone: 49-511-856 7598. Fax: 49-511-856 7697.

bovine apotransferrins were iron saturated as described by Herrington and Sparling (16).

For immunological detection, sera against the recombinant *A. pleuropneumoniae* TfbA protein and against porcine transferrin were raised in rabbits. Porcine convalescent-phase serum was obtained from pigs experimentally infected by aerosol with *A. pleuropneumoniae* AP205 (29).

Preparation of protein aggregates and determination of protein concentrations. *E. coli* transformants were grown to an optical density at 660 nm of ≈0.4; cells were induced to form inclusion bodies by the addition of isopropylthiogalactoside (IPTG; 1 mM, final concentration). Two hours after induction, bacteria were harvested by centrifugation, and aggregate protein was prepared as described previously (9). Isolated proteins were analyzed by sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis performed by the method of Laemmli (20), and concentration was determined by comparing the intensities of bands with those of a bovine serum albumin standard (Pierce Chemical Co., Rockford, Ill.) after Coomassie blue staining.

For binding studies, aggregated protein was dissolved in 3.5 M guanidine hydrochloride and diluted at least 100-fold in Tris-buffered saline (TBS)–Tween (15 mM Tris, 150 mM NaCl, 0.5% Tween 80, [pH 8]). TfbA protein prepared in this way was immunologically indistinguishable from wild-type protein and specifically bound iron-saturated porcine transferrin (9).

Biotinylation of TfbA protein. For biotinylation, TfbA protein was dissolved in nonreducing sample buffer (25), warmed to 37°C for 20 min, and purified in a Prepcell (Bio-Rad, Munich, Germany), and SDS was removed in an Extracti-Gel D detergent-removing column (Pierce). The protein was biotinylated via its lysine residues by the method of Bayer et al. (1) as described by Kessler (18). Biotinylated recombinant TfbA protein showed binding specific for iron-saturated porcine transferrin indistinguishable from that of nonbiotinylated protein.

Inhibition of transferrin binding by convalescent-phase serum. Enzyme-linked immunosorbent assay (ELISA) plates (Maxisorb; Nunc, Wiesbaden, Germany) were coated overnight at 4°C with 100 μl of recombinant TfbA protein (10 μg/ml diluted in coating buffer). All subsequent steps were done at room temperature; plates were incubated for 1 h with blocking buffer (TBS-Tween containing 0.5% gelatin) and washed three times with TBS-Tween. Serial twofold dilutions of porcine preimmune and convalescent-phase sera in blocking buffer were incubated for 1 h in the TfbA-coated plates. Subsequently the plates were washed and developed by using antitransferrin antibody, alkaline phosphatase conjugate, and *p*-nitrophenyl phosphate.

Preparation, detection, and characterization of transposon mutants. Transposon mutagenesis was performed with λTn*phoA* as described previously (23). Mutants were mapped by restriction enzyme analysis, and the exact site of insertion was determined by nucleotide sequence analysis (30) using a primer complementary to *phoA* (3). Transferrin binding of mutants was tested in a solid-phase assay. Briefly, *E. coli* transformants were grown to an optical density at 660 nm of ~0.5, induced with 100 μM IPTG for 2 h at 37°C, and collected by centrifugation. Pellets were resuspended in 1/10 volume of blocking buffer and exposed to three freeze-thaw cycles (−80°C-37°C). Serial twofold dilutions of these lysates were incubated for 1 h in ELISA plates coated with porcine transferrin (50 μg/ml in coating buffer at 4°C overnight). Plates were washed and developed with *p*-nitrophenyl phosphate (15 mM) in substrate buffer (1 M diethanolamine, 0.5 mM $MgCl_2$ [pH 9.8]). The optical density was measured at 405 nm in an ELISA plate reader (Dynatech MR5000; Dynatech Laboratories Inc., Alexandria, Va.).

Preparation and detection of random mutants. Random mutagenesis was performed in vitro on isolated *Nsi*I-*Kpn*I fragments, using hydroxylamine as the mutagen (6). After incubation for 18 h at 37°C, fragments were religated into pTF205/E2 cut with *Nsi*I-*Kpn*I, and the plasmids were transformed into *E. coli* HB101. Transformants were plated and screened in a colony transferrin-binding assay. Briefly, *E. coli* transformants were cultivated on Luria agar plates with ampicillin at 37°C overnight, transferred onto nitrocellulose, and induced on Luria agar plates with a final concentration of 1 mM IPTG for 1 h at 37°C. Cell material not firmly bound to the membrane was washed off with TBS. Filters were blocked by incubation in blocking buffer for 1 h and washed three times in TBS-Tween. For analysis of transferrin-binding ability, 50 μg of porcine transferrin per ml in blocking buffer was added. Bound transferrin was detected immunologically with rabbit anti-porcine transferrin antiserum and alkaline phosphatase-labeled conjugate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.). Blots were developed with Nitro Blue Tetrazolium (100 μg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP; 50 μg/ml) in substrate buffer (100 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$ [pH 9.5]). All incubation steps were performed for 1 h at room temperature. All components were diluted in blocking buffer. Colonies with decreased transferrin-binding ability were investigated in an additional colony blot using convalescent-phase serum. Transformants expressing an immunologically active TfbA protein with decreased transferrin binding were characterized further in a competitive transferrin-binding assay and subsequent nucleotide sequence analysis (30).

Competitive transferrin-binding assay. To investigate transferrin-binding abilities of the TfbA mutant proteins, ELISA plates (Immunolon II; Dynatech Laboratories) were coated with porcine transferrin, blocked, and washed as described above. Serial twofold dilutions of the solubilized regular and mutant TfbA protein preparations in blocking buffer (100 μl per well containing 100 μg/ml) were incubated for 1 h in the transferrin-coated plates. Subsequently the plates were washed, 100 μl of biotinylated TfbA was added, and the plates were developed as described above. The 50% inhibition values of TfbA mutations were determined as multiples of the 50% inhibitory amount of regular (nonmutated) recombinant TfbA protein.

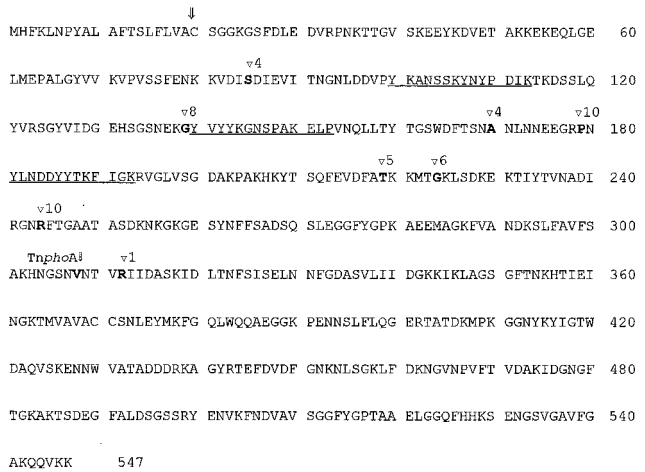

FIG. 1. Amino acid sequence of the TfbA protein of *A. pleuropneumoniae* serotype 7. The arrow marks the first residue of the mature protein (C). Arrowheads indicate the locations and designations of point mutations. Transferrin-binding domains detected in the solid-phase transferrin-binding assay (Fig. 2) are underlined. The arrow labeled with Tn*phoA* indicates the location of the largest TfbA-PhoA fusion still binding transferrin.

Peptide synthesis and transferrin-binding test. To more accurately localize transferrin-binding regions of the TfbA protein, 16-mer peptides starting at each amino acid of the amino-terminal half of TfbA were synthesized as described by Frank (8), using a multiple-peptide synthesizer (AMS422; Abimed Analysentechnik, Langenfeld, Germany), thus allowing peptides (~20 nM per spot) to be directly coupled onto a nitrocellulose sheet (9 by 13 cm). Transferrin binding of the peptides was analyzed in a blot assay established in analogy to antibody-binding assays (27) and the colony transferrin-binding assay described above. Nonspecific binding was blocked by incubation with casein-based blocking buffer (blocking reagent; Genosys Biotechnologies Inc., Cambridge, England) diluted in TBS-Tween with 5% sucrose overnight. After being washed with TBS-Tween (three times for 5 min each), the blots were incubated with 50 μg of porcine or bovine transferrin per ml in casein-based blocking buffer for 3 h at room temperature. Transferrin bound to peptides was detected (i) by immunoblotting with porcine transferrin, antibody, and alkaline phosphatase-labeled conjugate, (ii) by autoradiography after incubation with ^{59}Fe-loaded transferrin, and (iii) by using biotinylated transferrin.

RESULTS

The amino-terminal half of TfbA contains the transferrin-binding region(s) and is not detected by convalescent-phase serum. The Tn*phoA* mutant which expressed the fusion protein with the smallest TfbA portion and still recognized porcine transferrin in the solid-phase assay contained the first 289 amino acids of the mature protein (Fig. 1). In addition, six random mutations resulting in decreased transferrin binding all mapped in the amino-terminal portion of the TfbA protein as determined by nucleotide sequence analysis (Fig. 1; Table 1). Convalescent-phase serum did not detect the smallest transferrin-binding mutant, and it did not interfere with transferrin binding of the intact TfbA protein any differently than did porcine preimmune serum.

Transferrin binding is mediated by at least three distinct parts of the TfbA polypeptide. Overlapping 16-mer peptides were analyzed in a spot blot assay using the three different detection methods described above. Consistently the spots at positions D4 to D6, E19 to E21, and G9 to G12, representing amino acids 81 to 94, 121 to 134, and 162 to 174, were shown to bind porcine transferrin (Fig. 2; Table 2). All three regions also recognized bovine transferrin (data not shown). In all

TABLE 1. Locations of amino acid changes in purified TfbA mutant proteins[a] with decreased transferrin-binding ability

Designation	Location(s)	Amino acid change
$TfbA_{mut1}$	291 and 293	Silent and R→W
$TfbA_{mut4}$	47 and 151	S→L and A→V
$TfbA_{mut5}$	200	T→I
$TfbA_{mut6}$	205	G→D
$TfbA_{mut8}$	120	G→E
$TfbA_{mut10}$	160 and 225	P→L and R→C

[a] Products of *tfbA* genes after hydroxylamine mutagenesis.

TABLE 2. Designations, positions, and lengths of transferrin-binding domains of TfbA protein of *A. pleuropneumoniae* serotype 7

Domain	Position in mature protein (aa[a])	Length (aa)
1	81–94	14
2	121–134	14
3	162–174	13

[a] aa, amino acids.

cases, turns and β-sheet structures (no α helices) are predicted for these peptides (Fig. 3); the hydropathicity profile for all three peptides contains a weakly hydrophilic part and a carboxy-terminal hydrophobic part which is coupled onto the nitrocellulose membrane. Comparison of the three TfbA isoforms from *A. pleuropneumoniae* serotypes 1, 5, and 7 shows no primary amino acid agreement in the domains enclosing residues 81 to 94 and 162 to 174; however, the domain containing residues 121 to 134 is highly conserved among the three isoforms (Fig. 4), having 71% amino acid sequence identity, compared with 36% over the entire amino-terminal half of the molecule. In addition, a highly homologous amino acid sequence was located in the small transferrin-binding proteins of *Neisseria gonorrhoeae* (22) and *N. meningitidis* (21); also, a similar peptide was detectable in the Tbp2 protein of *Haemophilus influenzae* (13) (Fig. 4).

Transferrin binding by the TfbA mutants is quantitated in Table 3.

DISCUSSION

In this study, we have investigated the interaction between the *A. pleuropneumoniae* serotype 7 TfbA protein with porcine transferrin. The TfbA molecule is a 60-kDa protein which is expressed under iron-limiting growth conditions only (9). Three isoforms of the protein have been identified in different *A. pleuropneumoniae* serotypes; they are molecules with a variable amino-terminal half and a conserved carboxy-terminal half (2, 10). The protein is detected by convalescent-phase serum, and the recombinant TfbA protein has been shown to induce a serotype-specific protective immune response (29). Similar observations have been reported for *N. gonorrhoeae* and *N. meningitidis*, which likewise express a transferrin-binding protein with a molecular mass of about 60 kDa (21, 22). As in *A. pleuropneumoniae*, this protein has been shown to induce a serotype-specific protective immune response (7, 14).

We hypothesized that transferrin binding by the different *A. pleuropneumoniae* TfbA isoforms might be due to similar primary and/or secondary structures. Thus, we set out to define the active binding region of the TfbA protein of *A. pleuropneumoniae* serotype 7.

Our finding that the amino-terminal half of the TfbA molecule is responsible for transferrin binding was surprising, since this part of the molecule is highly variable among the three isoforms. In addition, this part of the molecule is not recognized by convalescent-phase serum, which could be advantageous for the pathogen since it apparently prevents antibodies directed against the highly immunogenic carboxy-terminal part of the molecule from interfering with transferrin binding, and such antibodies are thus unlikely to strongly interfere with the iron uptake mechanism.

To confirm the localization of transferrin-binding activity in the amino-terminal half of the molecule, we characterized six random TfbA mutants with decreased transferrin-binding ability by nucleotide sequence analysis. All mutations mapped to the amino-terminal half of the TfbA protein. A closer analysis of the mutants showed that they could be classified into two groups: mutants with moderately ($TfbA_{mut1}$, $TfbA_{mut4}$, and $TfbA_{mut5}$) and mutants with severely ($TfbA_{mut6}$, $TfbA_{mut8}$, and $TfbA_{mut10}$) decreased binding activity. The first class of mutants showed amino acid changes which do not cause an alteration of predicted secondary structure (4) but cause a rather slight change of the hydrophobicity profile (19). In contrast, the second class of mutants ($TfbA_{mut6}$, $TfbA_{mut8}$, and

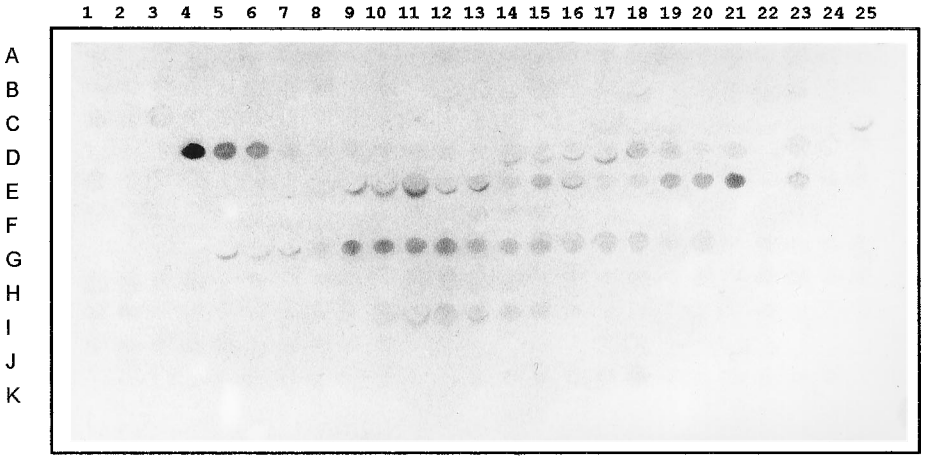

FIG. 2. Solid-phase transferrin binding of synthetic peptides, using biotinylated porcine transferrin and streptavidin-phosphatase for detection. Spots at positions D4 to D6 (domain 1), E19 to E21 (domain 2), and G9 to G12 (domain 3) reproducibly reacted with porcine transferrin.

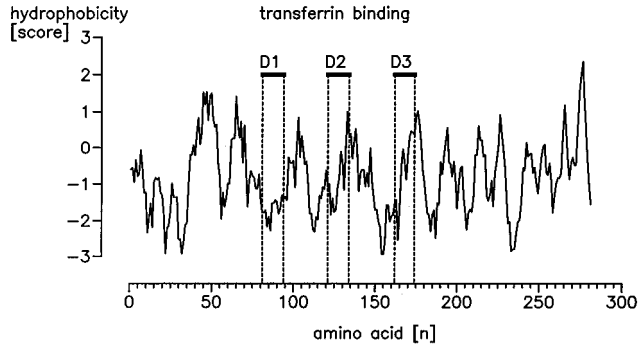

FIG. 3. Hydrophobicity profile of the TfbA protein from *A. pleuropneumoniae* serotype 7. D1, D2, and D3 represent the transferrin-binding domains 1, 2, and 3 which were detected in the solid-phase transferrin-binding assay.

$TfbA_{mut10}$) have predicted secondary structure alterations, since a strong helix breaker (G or P) has been changed in all cases.

The localization of mutants with single amino acid changes over a range of more than 170 amino acids as well as the existence of different classes of mutants indicated that more than one region of TfbA is responsible for transferrin binding. To more closely define these regions, overlapping 16-mer peptides were synthesized and analyzed in a solid-phase binding assay. Three regions with a length of 13 or 14 amino acids each were identified. All three regions are predicted to be devoid of α-helical structures and are located between two strong helix-breaking residues (G or P). The importance of this structure is emphasized by the point mutations in $TfbA_{mut8}$ and $TfbA_{mut10}$, both deleting a helix breaker from the primary sequence and causing severely impaired binding activity. The possible significance of our findings in the peptide-binding approach was emphasized by the observation that the second domain (residues 121 to 134) was highly conserved among the three TfbA isoforms of *A. pleuropneumoniae* and in addition that a homologous sequence was found to be present in the otherwise totally distinct small transferrin-binding proteins of *Neisseria* spp. (21, 22). This peptide sequence of *N. meningitidis* is located within a domain of 290 amino acids that has been shown previously to be responsible for transferrin binding (33). Further supporting our findings, a similar sequence was observed in the Tbp2 protein of *H. influenzae* (13).

The results show that transferrin binding by the *A. pleuropneumoniae* serotype 7 TfbA protein (i) is not a simple epitope-paratope-like interaction but involves at least three different portions of the TfbA protein, (ii) is not inhibited by convalescent-phase serum, and (iii) may be comparable to the transferrin binding of analogous proteins of other organisms.

A. pp. serotype 1	175	G Y V Y Y L G V T P S K E L P	189
A. pp. serotype 5	146	G Y V Y Y K G V H P S K E L P	160
A. pp. serotype 7	120	**G Y V Y Y K G N S P A K E L P**	134
N. gonorrhoeae	223	G Y I F Y H G D K P S R Q L P	237
N. meningitidis	161	G Y I F Y H G E K P S R Q L P	175
H. influenzae	131	G Y A Y Y F G N T T A S A L P	145

FIG. 4. Amino acid sequences homologous to the TfbA-derived transferrin-binding domain 2. *A. pp.* serotypes 1, 5, and 7 indicate the sequences derived from the TfbA proteins from *A. pleuropneumoniae* serotypes 1, 5, and 7. *N. gonorrhoeae*, *N. meningitidis*, and *H. influenzae* indicate sequences within the TfbA protein analogs of these organisms. The locations of the first and last amino acids of each peptide within the respective mature TfbA analog are given by the numbers before and after the sequence. Lines mark identical amino acids; colons mark functionally similar residues (I and V, hydrophobic; N, D, E, and Q, polar or negatively charged; K, R, and H, positively charged; Y and F, aromatic; A and S, small neutral).

TABLE 3. Transferrin binding by mutated recombinant TfbA protein of *A. pleuropneumoniae* serotype 7

Protein[a]	Multiple of TfbA	
	Mean[b]	SD
TfbA	1	
$TfbA_{mut1}$	14	±1
$TfbA_{mut5}$	12	±2
$TfbA_{mut6}$	46	±4
$TfbA_{mut8}$	66	±19

[a] The proteins derived by hydroxylamine mutagenesis are designated $TfbA_{mut1}$, $TfbA_{mut4}$, $TfbA_{mut5}$, $TfbA_{mut6}$, $TfbA_{mut8}$, and $TfbA_{mut10}$. Mutants $TfbA_{mut4}$ and $TfbA_{mut10}$ were not characterized in detail since they were shown to have two amino acid changes (Table 1); single experiments indicated $TfbA_{mut4}$ reacting similarly to $TfbA_{mut1}$ and $TfbA_{mut5}$ and $TfbA_{mut10}$ binding similarly to $TfbA_{mut6}$ and $TfbA_{mut8}$.

[b] Arithmetical mean of three experiments, each done in duplicate; values represent the multiples of mutant TfbA protein necessary to inhibit transferrin binding of biotinylated TfbA protein by 50%.

ACKNOWLEDGMENTS

This work was supported by grant Ge 522/2-1 from the Deutsche Forschungsgemeinschaft. Katrin Strutzberg is a fellow of the Graduiertenkolleg IIIGK-Re 592/7-1 funded by the Deutsche Forschungsgemeinschaft.

REFERENCES

1. **Bayer, E. A., M. Wilchek, and E. Skutelsky.** 1976. Affinity cytochemistry: the localization of lectin and antibody receptors on erythrocytes via the avidin-biotin-complex. FEBS Lett. **68:**240–244.
2. **Bunka, S., A. A. Potter, and G.-F. Gerlach.** 1995. Cloning and sequencing of the transferrin-binding protein genes of *Actinobacillus pleuropneumoniae*, biotype 1-serotype 5 and biotype 2-serotype 2. Unpublished; GenBank accession no. Z46774.
3. **Chang, C. N., W.-J. Kuang, and E. Y. Chen.** 1986. Nucleotide sequence of the alkaline phosphatase gene of *Escherichia coli*. Gene **44:**121–125.
4. **Chou, B. Y., and G. D. Fasman.** 1978. Empirical predictions of protein conformation. Annu. Rev. Biochem. **47:**251–276.
5. **Devenish, J., S. Rosendal, and J. T. Bossé.** 1990. Humoral antibody response and protective immunity in swine following immunization with the 104-kilodalton hemolysin of *Actinobacillus pleuropneumoniae*. Infect. Immun. **58:** 3829–3832.
6. **Eichenlaub, R.** 1984. Isolation of suppressible mutations by hydroxylamine mutagenesis in vitro, p. 106–110. *In* A. Pühler and K. N. Timmis (ed.), Advanced molecular genetics. Springer-Verlag, Berlin.
7. **Ferron, L., C. M. Ferreios, M. T. Criado, and M. Pintor.** 1992. Immunogenicity and antigenic heterogeneity of a human transferrin-binding protein in *Neisseria meningitidis*. Infect. Immun. **60:**2887–2892.
8. **Frank, R.** 1992. Spot-synthesis: an easy technique for the positionally addressable, parallel chemical synthesis on a membrane support. Tetrahedron **48:**9217–9232.
9. **Gerlach, G.-F., C. Anderson, A. A. Potter, S. Klashinsky, and P. J. Willson.** 1992. Cloning and expression of a transferrin-binding protein from *Actinobacillus pleuropneumoniae*. Infect. Immun. **60:**892–898.
10. **Gerlach, G.-F., S. Klashinsky, C. Anderson, A. A. Potter, and P. J. Willson.** 1992. Characterization of two genes encoding distinct transferrin-binding proteins in different *Actinobacillus pleuropneumoniae* isolates. Infect. Immun. **60:**3253–3261.
11. **Geysen, H. M., S. J. Rodda, T. J. Mason, G. Tribbick, and P. G. Schoofs.** 1987. Strategies for epitope analysis using peptide synthesis. J. Immunol. Methods **102:**259–274.
12. **Gonzalez, G. C., D. L. Caamano, and A. B. Schryvers.** 1990. Identification and characterization of a porcine-specific transferrin-receptor in *Actinobacillus pleuropneumoniae*. Mol. Microbiol. **4:**1173–1179.
13. **Gray-Owen, S. D., S. Loosmore, and A. B. Schryvers.** 1995. Identification and characterization of genes encoding the human transferrin-binding proteins from *Haemophilus influenzae*. Infect. Immun. **63:**1201–1210.
14. **Griffiths, E., P. Stevenson, and A. Ray.** 1990. Antigenic and molecular het-

erogeneity of the transferrin-binding protein of *Neisseria meningitidis*. FEMS Microbiol. Lett. **69:**31–38.
15. **Hensel, A., R. Pabst, S. Bunka, and K. Petzoldt.** 1994. Oral and aerosol immunization with viable or inactivated *Actinobacillus pleuropneumoniae* bacteria: antibody response to capsular polysaccharides in broncoalveolar lavage fluids (BALF) and sera of pigs. Clin. Exp. Immunol. **96:**91–97.
16. **Herrington, D. A., and F. P. Sparling.** 1985. *Haemophilus influenzae* can use human transferrin as a sole source for required iron. Infect. Immun. **48:**248–251.
17. **Higgins, R., S. Lariviére, K. R. Mittal, G. P. Martineau, P. Rousseau, and J. Cameron.** 1985. Evaluation of a killed vaccine against porcine pleuropneumoniae due to *Haemophilus pleuropneumoniae*. Can. Vet. J. **26:**3125–3129.
18. **Kessler, D.** 1992. Nonradioactive labeling and detection of biomolecules, p. 92–93. Springer-Verlag, Berlin.
19. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157:**105–132.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
21. **Legrain, M., V. Mazarin, S. Irwin, B. Bouchon, M. J. Quentin-Millet, E. Jacobs, and A. B. Schryvers.** 1993. *N. meningitidis* tbp1 and tbp2 genes encoding transferrin binding proteins 1 and 2. Unpublished; GenBank accession no. Z15130.
22. **Lissolo, L., G. Maitre-Wilmotte, P. Dumas, M. Mignon, B. Danve, and M.-J. Quentin-Millet.** 1995. Evaluation of transferrin-binding protein 2 within the transferrin-binding protein complex as a potential antigen for future meningococcal vaccines. Infect. Immun. **63:**884–890.
23. **Manoil, C., and J. Beckwith.** 1985. Tn*phoA*: a transposon probe for protein export signals. Proc. Natl. Acad. Sci. USA **82:**8129–8133.
24. **Mazurier, J., and G. Spik.** 1980. Comparative study of the iron-binding properties of human transferrins. I. Complete and sequential iron saturation and desaturation of the lactotransferrin. Biochim. Biophys. Acta **629:**399–408.
25. **Morton, D. J., and P. Williams.** 1990. Siderophore-independent acquisition of transferrin-bound iron by *Haemophilus influenzae* type b. J. Gen. Microbiol. **136:**927–933.
26. **Niven, D. F., J. Donga, and F. S. Archibald.** 1989. Responses of *Haemophilus pleuropneumoniae* to iron restriction: changes in the outer membrane protein profile and the removal of iron from porcine transferrin. Mol. Microbiol. **3:**1083–1089.
27. **Raupach, B., and M. A. Schmidt.** 1994. Elucidation of linear epitopes of pertussis toxin using overlapping synthetic decapeptides: identification of a human B-cell determinant in the S1 subunit indicative of acute infections. Microb. Pathog. **17:**213–226.
28. **Ricard, M. A., F. S. Archibald, and D. F. Niven.** 1991. Isolation and identification of a putative porcine transferrin receptor from *Actinobacillus pleuropneumoniae* biotype 1. J. Gen. Microbiol. **137:**2733–2740.
29. **Rossi-Campos, A., C. Anderson, G.-F. Gerlach, S. Klashinsky, A. A. Potter, and P. J. Willson.** 1992. Immunization of pigs against *Actinobacillus pleuropneumoniae* with two recombinant protein preparations. Vaccine **10:**512–518.
30. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
31. **Schryvers, A. B., and L. J. Morris.** 1988. Identification and characterization of the transferrin receptor from *Neisseria meningitidis*. Mol. Microbiol. **2:**281–288.
32. **Sebunya, T. N. L., and K. R. Saunders.** 1983. *Haemophilus pleuropneumoniae* infection in swine: a review. J. Am. Vet. Med. Assoc. **182:**1331–1337.
33. **Vonder Haar, R. A., M. Legrain, H. V. J. Kolbe, and E. Jacobs.** 1994. Characterization of a highly structured domain in Tbp2 from *Neisseria meningitidis* involved in binding to human transferrin. J. Bacteriol. **176:**6207–6213.