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Identification and characterization of the TonB region and its role in transferrin-mediated iron acquisition in *Haemophilus parasuis*

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Abstract

Haemophilus parasuis is the causative agent of Glässer's disease, which is responsible for considerable economic losses in the pig-rearing industry. The aim of the study reported here was the identification, sequencing and molecular characterization of the TonB region that includes *tonB*, *exbBD*, and *tbpBA* genes in *H. parasuis.* In addition, two fusion proteins were generated. One of them (pGEX-6P-1-GST-TbpB) contained the first 501 amino acids of *H. parasuis* TbpB protein, while the second (pBAD-Thio-TbpB-V5-His) included the first 102 amino acids of *H. parasuis* TbpB N-terminus domain. A panel of 14 hybridomas secreting monoclonal antibodies was raised against the two recombinant TbpB fusion proteins. Furthermore, to assess whether the expression of the *H. parasuis* ExbB, TbpB, and TbpA proteins was upregulated under conditions of restricted availability of iron, a rabbit polyclonal antibody against *H. parasuis* TbpB-His fusion protein was produced. A rabbit polyclonal antibody against serotype 7 of *Actinobacillus pleuropneumoniae* ExbB and TbpA proteins was also used for the detection of the homologous proteins in *H. parasuis*. Overall, the data indicate that *H. parasuis*, like other members of the *Pasteurellaceae* family, possesses the genetic elements of the TonB region for iron acquisition and the transferrin-binding proteins encoded under this region are upregulated under restricted iron availability.

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Keywords: *Haemophilus parasuis*; Iron acquisition; TonB region; Transferrin-binding protein

1. Introduction

Haemophilus parasuis is a non-motile, nicotinamide adenine dinucleotide (NAD)-dependent, Gram-negative rod of the family *Pasteurellaceae* (belonging to the γ subclass of the class *Proteobacteria*). This species is the causative agent responsible for Glässer's disease, a porcine fibrinous polyserositis, polyarthritis, meningitis and stress-associated disease of young pigs. It is an emerging challenge in the pig-rearing industry worldwide, often associated with significant financial losses, and has high morbidity and mortality rates [1].

To date, only a few virulence factors have been reported in relation to the pathogenicity of Glässer's disease. As in other Gram-negative microorganisms, lipopolysaccharide is responsible for endotoxic shock and its side effects [2]. The presence of capsule frequently correlates with virulence [3]. Certain outer membrane profiles [4,5], fimbriae [6] or neuraminidase (sialidase) [7,8] have also been linked to the virulence of this microorganism.

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Most living microorganisms require iron for growth and survival. In mammalian tissues, most of iron is transported by serum proteins, such as transferrin, hemoglobin and lactoferrin [9]. For the access to these limited resources of iron, pathogenic bacteria can either synthesize low molecular weight iron ligands, named siderophores, or use high-affinity iron uptake systems, such as transferrin-binding proteins, which are often upregulated under low iron conditions.

Morton and Williams proposed the first model of iron acquisition in *H. parasuis* [10], and showed that this bacterium could bind porcine transferrin. Later on, Charland et al. [11], assigned this function to two potential porcine transferrin binding polypeptides, which could specifically bind iron from porcine transferrin, but not from porcine lactoferrin.

The purpose of the work being reported here was the identification and characterization of the TonB region genes involved in transferrin iron uptake and their respective proteins in *H. parasuis*. To that end, two TbpB fusion recombinant proteins of *H. parasuis*, along with a panel of 14 monoclonal antibodies (mAbs) and a polyclonal antibody (pAb) anti-TbpB were produced. Furthermore, two cross-reactive pAbs against *Actinobacillus pleuropneumoniae* serotype 7 ExbB and TbpA proteins were also used to detect the expression of these proteins under iron-replete and iron-restricted conditions. The data demonstrate for the first time the presence of the TonB region in *H. parasuis* and that the proteins encoded by these genes are upregulated under iron-poor conditions. It also proved possible to show that the N-terminus domain of the *H. parasuis* TbpB protein is an immunodominant region, similar to what has been reported for the N-terminal domain of *Neisseria meningitidis* TbpB protein [12].

2. Materials and methods

2.1. Bacterial strains and growth conditions

The strains and plasmids used in this study are shown in Table 1. *H. parasuis* and *A. pleuropneumoniae* reference strains were cultured onto chocolate agar plates (BioMérieux) and incubated for 24 h at 37 °C. *Escherichia coli* BL21 and TOP10 cells were grown in Luria Bertani (LB) medium supplemented with ampicillin (100 μg ml^{-1}). The iron chelator, 2.2 dipyridyl (100 μM) (Sigma) was added to 0.025% NAD-supplemented PPLO broth (Difco) so as to attain restricted iron availability.

2.2. PCR amplification and recombinant DNA techniques

For the extraction of bacterial genomic DNA, the strains were grown as described above and harvested with PPLO broth. The cells were centrifuged at 3000*g* for 10 min at 4 °C, washed twice with TE buffer (10 mM Tris–HCl [pH 8.0], 1 mM EDTA), and finally lysed in buffer (0.5 M EDTA [pH 8.0], 20% SDS, 50 mg ml^{-1} of proteinase K) for 1 h at 55 °C. For RNA removal, DNAse free RNAse (100 μg ml^{-1}, Roche) was added and incubated for 20 min at 37 °C. Proteins were removed by adding 0.25% phenol equilibrated in TE buffer (pH 7.8). The DNA was then purified by repeated chloroform–isopropanol (24:1) extraction, precipitated by adding 0.1 vol of 3 M sodium acetate (pH 5.2), and 1 vol of isopropanol. The DNA was washed twice in 70% ethanol, allowed to dry and resuspended in double-distilled water.

The PCR mixture consisted of 3 μl of a sample containing genomic DNA, 1 U of *Taq* DNA polymerase (Roche Diagnostics), 5 μl of 10× PCR amplification buffer (10 mM Tris–HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3) (Roche Diagnostics), 1 μl of each primer (20 μM), 0.5 μl of dNTPs (25 mM each), and double-distilled water to a final volume of 50 μl. After optimizing PCR conditions, the reaction was performed in a DNA thermal cycler (Eppendorf Mastercycler Gradient, Hamburg, Germany). Primers used for amplification and sequencing of TonB region are shown in Table 2.

The DNA fragments resulting from digestion of plasmid (pGEX-6P-1-GST) with endonuclease restriction enzymes and PCR products were separated on agarose gels, cut from the gel under a UV transilluminator and then purified with a QIAquick gel extraction kit (QIAGEN).

2.3. DNA sequencing

For the complete sequencing of the genes included in *H. parasuis* TonB region, primers listed in Table 2 were used. DNA sequencing was performed at "Sistemas Genómicos" DNA Sequencing Core Facility in Valencia (Spain) using a capillary Beckman CEQ 2000 XL sequencer in accordance with the manufacturer's instructions. Both strands of the each gene included in TonB region were sequenced. The sequences obtained were analyzed using the BLAST computer program at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST). The computer software package DNAMAN version 5.2 (Lynnon Biosoft) was also used for designing the primers and alignment of the sequences.

2.4. Cloning and expression of recombinant glutathione S transferase (GST)-tagged TbpB fusion protein

The first 1503 bp (501 amino acids, AAs) of *H. parasuis* serotype 5 *tbpB* gene were amplified using the primers mAB F and mAB R that contained the restriction sites for *Eco*RI and *Sal*I, respectively (Table 2). The specific amplified PCR product was cut with the same

Table 1
Strains and plasmids used in this study

Strains and plasmids	Description	Source
H. parasuis	Reference serotype	
No. 4	Serotype 1	Kielstein, P.
SW140	Serotype 2	Kielstein, P.
SW114	Serotype 3	Kielstein, P.
SW124	Serotype 4	Kielstein, P.
Nagasaki	Serotype 5	Kielstein, P.
131	Serotype 6	Kielstein, P.
174	Serotype 7	Kielstein, P.
C5	Serotype 8	Kielstein, P.
D74	Serotype 9	Kielstein, P.
H 555	Serotype 10	Kielstein, P.
H 465	Serotype 11	Kielstein, P.
H 425	Serotype 12	Kielstein, P.
84-17975	Serotype 13	Kielstein, P.
84-22113	Serotype 14	Kielstein, P.
84-15995	Serotype 15	Kielstein, P.
E. coli		
DH5αF′	F′/*end*A1 *hsd*R17 ($r_k^- m_k^-$ *sup*E44 *thi*-1 *rec*A1 *gyr*A (Nal^r *rel*A1 Δ(*lac*ZYA–*arg*F)U169 *deo*R [ϕ 80d*lac*Δ(lacz)M15]	Amersham
BL21	F-*ompT* [*lon*] $hsdS_B(r_{B-} m_{B-})$ DE3	Amersham
TOP10	F$^-$*mcrA*Δ(*mrr*-*hsd*RMS-*mcr*BC) ϕ80*lac*X74 *rec*A1 *deo*R ara*D139* Δ(*araleu*)7697 *gal*U *gal*K *rps*L (Str^r) *end*A1 *nup*G	Invitrogen
Plasmids		
pCR 2.1-TOPO	Cloning vector for the insertion of *Taq* polymerase-amplified PCR products	Invitrogen
pBAD-Thio-V5-His-TOPO	*E. coli* expression vector carrying the thioredoxin gene, an ampicillin resistance determinant, V5 epitope and 6X His-tag for the preparation of hexahistidine-tagged N-terminal TbpB fusion proteins	Invitrogen
pGEX-6P-1-GST	*E. coli* expression vector carrying an ampicillin resistance determinant and a GST tag for the preparation of GST-TbpB fusion proteins	Amersham

restricted enzymes as the vector, excised from the agarose gel and purified using the QIAquick gel extraction kit.

The pGEX-6P-1-GST plasmid was digested with *Eco*RI and *Sal*I restriction enzymes and the digested PCR product was cloned directionally and in-frame into the *Eco*RI and *Sal*I sites of this expression vector, and ligated overnight at 16 °C using T4 DNA ligase (Roche Diagnostics). The ligation mixture was later used to transform electrocompetent *E. coli* DH5αF′ cells. A clone containing the recombinant plasmid with the appropriate insert was isolated and purified using a QIAprep Spin Miniprep kit (QIAGEN) and both strands of the insert were sequenced using the primers (5′ pGEX, mAB F, mAB R, and 3′ pGEX, Table 1).

The pGEX-6P-1-GST-TbpB (GST-TbpB) construction was transformed into *E. coli* BL21 for the expression of the recombinant GST-TbpB fusion protein and one transformant was grown in LB medium supplemented with ampicillin (100 μg ml^{-1}) overnight at 37 °C. Thereafter, 1/100 vol of the overnight culture was used to inoculate 1 l of LB medium supplemented with ampicillin until the optical density $(OD)_{600}$ reached 0.6. Next, the expression of the GST-TbpB fusion protein was induced by the addition of isopropylthiogalactoside (IPTG) at different concentrations (ranging from 0.1 to 1 mM). Likewise, several temperatures (30 and 37 °C) and times of incubation (ranging from 30 min to 2 h) were tested to find out the optimal expression conditions of the GST-TbpB fusion protein. A control culture of *E. coli* BL21 cells harboring only the cloning vector without the *tbpB* gene cassette was produced, and non-transformed *E. coli* BL21 control was also induced under the same conditions as those used for the induction of the GST-TbpB fusion protein. Glutathione–sepharose affinity chromatography (Amersham) was used for the purification of the GST-TbpB fusion protein following the manufacturer's instructions. The

Table 2
Primers used for the amplification and sequencing of the *H. parasuis* TonB region

Primer	Sequence	Reference or source
TonB F (-51)	5′ AAA TAA ACG ATA ATG ATT TT 3′	This study
BA7 (581)	5′ CAA TGG ATC CAT TTT ATC TTC TTC AGG C 3′	[18]
ExbB R (1407)	5′ TTA TTT ATT TTC TCC ATA GTG ATG 3′	This study
ExbD F (1431)	5′ ATG GCA TTT GGA AGT TTT GAT AAA 3′	This study
ExbD R (1831)	5′ TTA TTT ACT AGG TTG AGT CAC AAA 3′	This study
ExbDTbpB (1827)	5′ CAA CCT AGT AAA TAA AAG CA 3′	This study
RE1 (1863)	5′ AAG TTT AAA ATG CAT ATT GC 3′	[18]
tbpB55 (1868)	5′ ATG CAT TTT AAA CTT AAT CCC 3′	[31]
TbpB INI P1 (2518)	5′ GGT AAT GGT AAA AAA GGT GAT 3′	This study
RTCB 55 BIS (3511)	5′ CAC GGA AAT GCG GTA TTT AG 3′	This study
tbpB33 (3622)	5′ CGT TTT GCA CCA AAG ACA GCG 3′	[31]
tbpA 55 (3693)	5′ TTA GCC TTG CTC TTC TTA GCC 3′	[31]
TbpA 55 P1 (4235)	5′ TGG TTA AAC TCA CTT GCT TTT 3′	This study
TbpA 55 1D (4774)	5′ ACC ATC GGA TTT ACT TAG AAC 3′	This study
TbpA 33 P2 (5468)	5′ ATC TCT CAG AGC GAT GTA GTG ATT 3′	This study
tbpA 33 (6460)	5′ AAG CTT GAA ACT AAG GTA CTC TAA 3′	[31]
TOPO TbpB F (3)	5′ CAT TTT AAA CTT AAT CCC TAT GC 3′	This study
TOPO TbpB R (309)	5′ AGT CTC GTG ATT TGG TAT 3′	This study
mAB F (1)	5′ CCG GAA TTC ATG CAT TTT AAA CTT AAT CCC TAT GCG 3′	This study
mAB R (1504)	5′ GTC GTC GAC CTA GAG CGA AGC CAG CAT CTG AGG TTT 3′	This study
Trx F (665)	5′ TTC CTC GAC GCT AAC CTG 3′	Invitrogen
pBAD R (871)	5′ GAT TTA ATC TGT ATC AGG 3′	Invitrogen
5′ pGEX (869)	5′ GGG CTG GCA AGC CAC GTT TG GT G 3′	Amersham
3′ pGEX (1033)	5′ CCT CTG ACA CAT GCA GCT CCC GG 3′	Amersham

crude lysate, unbound fraction and eluted fraction of glutathion–sepharose affinity chromatography were analyzed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) to find the optimal purification conditions.

2.5. Cloning and expression of recombinant hexahistidine (6xHis)-tagged TbpB fusion protein

The first 306 bp (102 AAs) of the *H. parasuis* serotype 5 *tbpB* gene were amplified using the primers TOPO TbpB F and TOPO TbpB R (Table 2). The PCR product was cut from the agarose gel, purified, cloned into the pBAD-Thio-V5-His expression vector, and transformed into electrocompetent *E. coli* TOP10 cells. A clone containing the pBAD-Thio-TbpB-V5-His (TbpB-His) construction was sequenced using primers Trx F, TOPO TbpB F, and pBAD R (Table 2). Once the insert orientation was confirmed, one clone was selected for the expression of the recombinant TbpB-His fusion protein.

Briefly, *E. coli* TOP10 transformants containing the TbpB-His fusion protein were grown in LB medium under similar conditions of those described above. Arabinose in concentrations ranging from 0.02% to 0.2% was then added to the culture medium so as to induce the recombinant fusion protein. Various temperatures (30 and 37 °C) and times of incubation (30 min–2 h) were assessed to determine the optimal expression conditions of the TbpB-His fusion protein. *E. coli* TOP10 cells transformed with emptied plasmid pBAD-Thio-V5-His, and non-transformed *E. coli* TOP10 controls were induced under similar conditions to those previously used for induction of the TbpB-His fusion protein.

Nickel affinity chromatography (HIS-Select™ HC Nickel affinity gel, Invitrogen) was used for the purification of the TbpB-His fusion protein following the manufacturer's instructions. Crude extracts, unbound and eluted fractions were analyzed by SDS–PAGE to monitor the optimal conditions for expression and purification.

2.6. Production and characterization of mAbs and pAbs

Two groups of six 8- to 12-week-old female BALB/c mice each (Charles River, Barcelona, Spain) were immunized with two different types of recombinant TbpB antigens. The antigen was emulsified in incomplete Freund's adyuvant and intraperitoneally injected for all the immunizations, except for the last booster, for which the antigen was dissolved in sterile saline solution and given intravenously. Mice in group A received small pieces of minced Coomassie-blue stained electrophoresis gel containing the band corresponding to GST-TbpB recombinant fusion protein (87.3 kDa), whereas mice in group B were injected with minced pieces of Ponceau Red-stained nitrocellulose membrane containing nickel-affinity chromatography purified TbpB-His recombinant fusion protein (27.3 kDa). Three days after the final booster, the mice showing the highest Enzyme Linked

Immunosorbent Assay (ELISA) anti-TbpB titers were euthanized, and their spleens collected and used for the production of mAbs in accordance with the protocol described by Köhler and Milstein [13] with slight modifications [14]. At 12 days after fusion, indirect ELISA was performed to screen for hybridomas secreting mAbs using GST-TbpB and TbpB-His recombinant fusion proteins as antigens, coated at 10 μg ml^{-1} onto 96 well plates (Polysorp, Nunc). Bacterial cell lysate prepared from *E. coli* transformed with the plasmid without insert, and the purified fraction corresponding to Thio-V5-His recombinant protein were used as negative control in the ELISA to select only those hybridomas producing mAbs of the desired specificity. The reactivity of the mAbs supernatants was confirmed in immunoblotting using purified TbpB-His fusion protein and Thio-V5-His purified recombinant protein (negative control) in a miniblotter with manifold (Device 28SL; Immunetics Inc., Cambridge, Massachusetts). Finally, the hybridomas were cloned twice in 96 well plates by the limiting dilution method.

For the production of pAbs against the TbpB-His fusion protein, two three-month-old New Zealand rabbits (Charles River, Barcelona) were immunized in accordance with a protocol similar to that used for immunizing the mice. With the purpose of reducing background staining, the antiserum produced against purified TbpB-His was adsorbed three times through three steps of incubation for 6 h with *E. coli* TOP10 cells transformed with the emptied plasmid pBAD-Thio-V5-His. This antiserum was used for the detection of *H. parasuis* TbpB protein in immunoblotting.

Two additional adsorbed antisera, against *A. pleuropneumoniae* ExbB and TbpA, respectively, were kindly provided by Professor G.F. Gerlach (Institut für Mikrobiologie und Tierseuchen, Tierärztlische Hochschule, Hannover, Germany) that cross-reacted with the homologous proteins in *H. parasuis* and were used for detecting expression of these proteins.

The mice and rabbits used in this study were handled and cared for in accordance with European Animal Care guidelines. All animals were maintained in the animal facility of the School of Veterinary Medicine of the University of Leon with free access to water and food.

2.7. Growth conditions and preparation of proteins extracts from H. parasuis and A. pleuropneumoniae from whole-cell lysates

Whole-cell lysates (WCL) from *H. parasuis* and *A. pleuropneumoniae* were prepared from bacterial culture grown under iron-rich and iron-poor conditions. A single colony of *H. parasuis* or *A. pleuropneumoniae* was inoculated into 2.5 ml PPLO medium and incubated overnight at 37 °C. Then, 0.5 ml of the overnight culture were inoculated into 4.5 ml PPLO medium and incubated under agitation until the cell density reached 0.3–0.4 at OD_{660}. To create iron-restricted conditions, 100 μM of 2.2 dipyridyl was added to the culture medium and allowed to grow while it was shaken for 2 h. Finally, 1.5 ml of bacterial cell culture were harvested by centrifugation at 11,000*g* for 5 min, and the supernatant was removed. The protein concentration was estimated by the micro-BCA protein assay with a bovine serum albumin standard (Pierce). Five microliters of the cell suspension were mixed with 5 μl of 2× SDS sample buffer, boiled at 100 °C for 5 min and centrifuged. Samples were separated on 12% SDS–PAGE gel at 100 V during 1.5 h. A pre-stained molecular weight standard (Precision Plus Protein Dual Color Standards, Bio-Rad) was run in parallel on the same gel to verify the molecular weight of the proteins present in the sample.

2.8. Immunoblotting

SDS–PAGE gel was transferred to a nitrocellulose membrane and immunoblotting was performed according to standard procedures [15]. Non-specific binding sites of the nitrocellulose membrane were blocked by incubating the membranes for 1 h at room temperature in Tris saline buffer (TBS) (10 mM Tris, 150 mM NaCl [pH 7.1]) containing 5% of skimmed milk. The nitrocellulose membrane was incubated overnight at 4 °C with optimal dilutions of the following primary mAbs or pAbs: HRPO-labeled goat polyclonal anti-GST (Amersham), HRPO-labeled murine anti-V5 mAb (Invitrogen), and unlabeled rabbit polyclonal anti-*A. pleuropneumoniae* ExbB, and TbpA, and finally a rabbit polyclonal anti-*H. parasuis* TbpB generated during the work being reported here. The membrane was next washed 3 times with TBS-Tween 20 0.05%. The unlabeled primary rabbit pAb was detected with HRPO-conjugated goat anti-rabbit IgG (heavy + light) (Nordic Immunological Laboratories, Denmark), added to the membrane and incubated for 1 h at room temperature. After washing, the reaction was finally developed by the addition of Enhanced Chemiluminescent substrate for 1 min (Amersham) to the membranes [16].

2.9. Nucleotide sequence accession numbers

The accession numbers for the genes included in TonB region corresponding to *H. parasuis* serotypes 5 and 7 are the following: *H. parasuis* serotype 5 *tonB* gene (GenBank Accession No. AY818055), *H. parasuis* serotype 5 *exbB* gene (GenBank Accession No. AY818056), *H. parasuis* serotype 5 *exbD* gene (GenBank Accession No. AY818057), *H. parasuis* serotype 5 *tbpB* gene (GenBank Accession No. AY818058), *H. parasuis* serotype 5 *tbpA* gene (GenBank Accession No. AY818059), *H. parasuis* serotype 7 *tonB* gene (GenBank Accession No. AY818060), *H. parasuis* serotype 7 *exbB* gene

(GenBank Accession No. AY818051), *H. parasuis* serotype 7 *exbD* gene (GenBank Accession No. AY818052), *H. parasuis* serotype 7 *tbpB* gene (GenBank Accession No. AY818053), and *H. parasuis* serotype 7 *tbpA* gene (GenBank Accession No. AY818054).

3. Results

3.1. Presence of *tonB, exbB, exbD, tbpB, and tbpA* genes in H. parasuis and heterogeneity of *tbpB* gene

The *tonB*, *exbB*, *exbD*, *tbpB*, and *tbpA* genes were amplified by PCR from genomic DNA of *H. parasuis* with different forward and reverse pairs of primer combinations that were designed on the basis of homologous gene sequences in *A. pleuropneumoniae* (Table 2). Thus, *tonB*, *exbB*, and *exbD* genes were amplified together using primers TonB F and ExbD R, yielding a PCR product of 1891 bp. Using primers sense tbpB55 and antisense tbpB33, *tbpB* gene was amplified and a PCR product ranging from 1638 to 1779 bp was obtained in all reference strains of *H. parasuis*, except serotypes 1 and 11. This size difference in the PCR product indicates a significant degree of TbpB gene heterogeneity in *H. parasuis*. On the basis of the different amplified PCR *tbpB* product size using the pair of primers sense tbpB55 and antisense tbpB33, two distinct groups of *H. parasuis* could be established: group 1, included serotypes 2–5, 7, 12, 14 and 15 (1636 bp), group 2, comprised serotypes 6, 8, 9, 10 and 13 (1779 bp). Finally, *tbpA* gene was also amplified with primers sense tbpA55 and antisense tbpA33 rendering a PCR product of 1900 bp.

3.2. Sequence analysis of H. parasuis *TonB region and comparison with homologous genes of other members of the Pasteurellaceae family*

H. parasuis serotype 5 and 7 *tonB*, *exbB*, *exbD*, *tbpB* and *tbpA* genes were amplified, cloned in pCR2.1-TOPO vector, and finally sequenced. The nucleotide sequences of these genes were virtually translated into AAs sequences and compared with the homologous proteins of other members of the *Pasteurellaceae* family available in the GenBank database. Table 3 shows the comparison of the AA sequence of *H. parasuis tonB*, *exbB*, *exbD*, *tbpB*, and *tbpA* serotypes 5 and 7 with the homologous AA sequences of the following strains: *Actinobacillus suis* strain SO4 (GenBank Accession No. AY101604), and *A. pleuropneumoniae* serotype 7 (GenBank Accession No. Y17916 and U16017).

3.3. Organization of H. parasuis *TonB region*

The TonB region was sequenced with the purpose of contributing to better characterization and understanding of the iron uptake mechanisms in *H. parasuis*. These genes are organized in the layout shown in Fig. 1. Sequence analysis of the TonB region from *H. parasuis* revealed five Open Reading Frames (ORFs) encoding for the TonB, ExbB, ExbD, TbpB, and TbpA proteins. The first ORF corresponded to *tonB* gene and encoded a predicted product of 246 AAs. It extended from position 1–740 bp. Before the localization of the start codon (ATG), a sequence of 19 pb was identified and turned out to be a Fur Box (GATAATG-ATTTTCATTAAC). A putative promoter sequence was also located at position −35 (TTTCA) and −10 (GATAAT) upstream to the transcription origin. The −10 region overlapped with the Fur Box. A Shine-Dalgarno (SD) sequence (GAGGAA) was located before the start codon of the *exbB* gene. The second ORF (ExbB) encoded a product of 222 AAs, extending from positions 762–1430 bp of the TonB region. The stop codon (TAA) overlapped with the start codon of *exbD* gene. Moreover, a second SD sequence (GGAGAA) was also located before the start codon of *exbD* gene. The analysis of the sequence in this region revealed the existence of a third ORF (ExbD) of 136 AAs, which extended from positions 1430–1840. The stop codon (TAA) was located 28 bp upstream of the *tbpB* start codon. A third SD sequence (GGAGGC) located before the *tbpB* start codon, was also identified. The

Table 3
Description of the genes in the TonB region of *H. parasuis* serotype 5 with the proteins they encode and their identities with homologous proteins from other *Pasteurellaceae*

Gene[a]	Base pairs	Amino acids	Molecular mass (kDa)	Identity (%) of *H. parasuis* serotype 5 with	
				A. pleuropneumoniae	*A. suis* SO4
tonB[b]	167	55	5.6	100	100
exbB	669	222	24.7	99.5	99.5
exbD	411	136	15.1	99.3	99.5
tbpB	1638	545	59.6	70.5	57.6
tbpA	2702	899	102.0	99.6	93.0

[a] GenBank accession numbers are shown in Section 2.9.
[b] Partial sequence (from 565 to 738 bp).

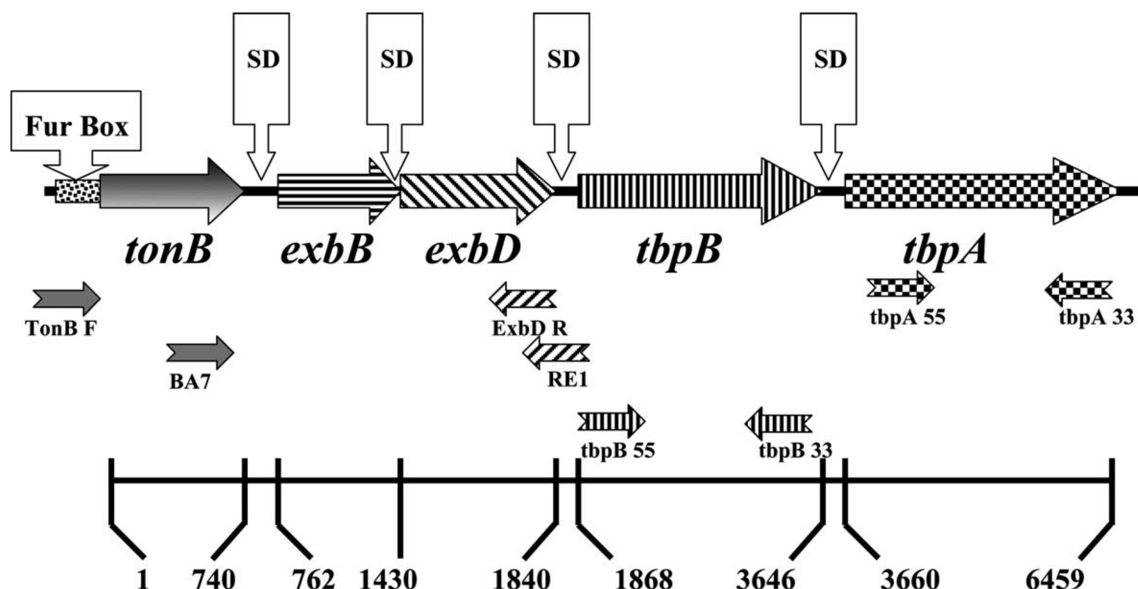

Fig. 1. Organization of the *H. parasuis* TonB region showing the length of genes, the primers used for the amplification, and the Shine-Dalgarno (SD) sequences.

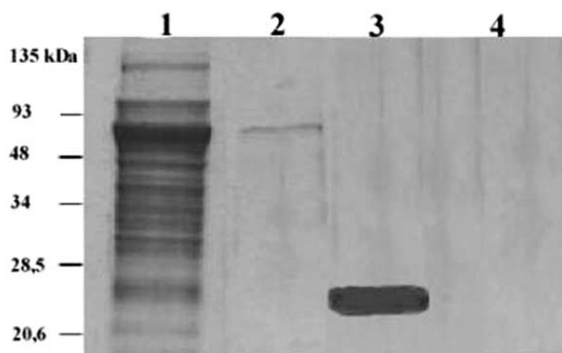

Fig. 2. Immunoblotting using a HRPO-labeled goat pAb anti-GST shows the expression of the GST-TbpB fusion protein induced with 1 mM IPTG, 30 °C, 2 h: lane 1, WCL of *E. coli* BL21 cells expressing the recombinant GST-TbpB from *H. parasuis* serotype 5; lane 2, GST-TbpB fusion protein cut from the SDS–PAGE used for immunization; lane 3, WCL of *E. coli* BL21 cells transformed with the emptied plasmid pGEX-6P-1-GST without insert, induced with IPTG 1 mM, 30 °C, 2 h; lane 4, non-transformed *E. coli* BL21 cells.

fourth ORF encoding for TbpB protein was located between positions 1868 and 3646 bp of TonB region and a stop codon (TAA) was identified 14 bp upstream of the *tbpA* start codon. A fourth SD sequence (AAG-GAA) before the *tbpA* start codon and a separation of 14 bp between the *tbpB* stop codon and *tbpA* start codon was also identified. Finally, a fifth ORF that included a region of 933 AAs (from positions 3660–6459) corresponding to TbpA protein was located. No evidence of other promoter sequences was found along the TonB region. To sum up, the TonB region is composed of five ORF encoding for TonB, ExbB, ExbD, TbpB, and TbpA proteins, which are separated by SD sequences.

3.4. Recombinant TbpB protein expression and purification

Two recombinant TbpB fusion proteins were prepared. One of them contained the complete sequence of the TbpB and was cloned into pGEX-6P-1-GST expression vector, while the second recombinant protein included the N-terminus domain of the TbpB protein and was cloned into pBAD-Thio-V5-His expression vector.

The optimal conditions for the expression of the GST-TbpB fusion protein were 1 mM IPTG, 30 °C for 2 h (Fig. 2). The molecular weight of the GST-TbpB fusion protein yielded a molecular mass of 82.3 kDa. Purification of this protein was not achieved under either denaturing or native conditions because it formed cytoplasmic inclusion bodies. Inclusion bodies were, however, solubilized after overnight incubation in 8 M urea buffer and the supernatant was run in SDS–PAGE. The molecular weight was further confirmed in Western blot with HRPO-labeled goat polyclonal anti-GST. For the immunization of BALB/c mice, the Coomassie-blue stained SDS–PAGE band corresponding to 82.3 kDa was cut and used.

To overcome the difficulty of TbpB purification, it was decided to prepare a second TbpB fusion protein containing only the N-terminus domain. This new recombinant fusion TbpB-His protein was induced in the presence of 0.075% arabinose, at 37 °C for 2 h (Fig. 3). This fusion protein was smaller than the GST-TbpB and yielded a molecular mass of 27.3 kDa, confirmed using HRPO-labeled murine anti-V5 mAb. Unlike the GST-TbpB protein, the TbpB-His protein did not form inclusion bodies and was affinity purified

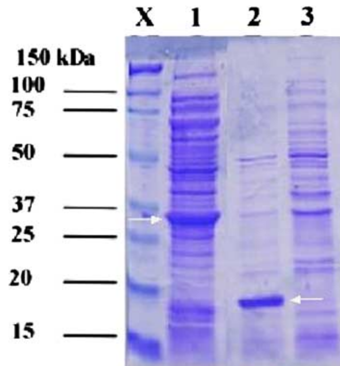

Fig. 3. SDS–PAGE depicts the expression TbpB-His fusion protein from *H. parasuis* serotype 5. Lane X, molecular weight marker ranging from 15 to 150 kDa (Biorad); lane 1, WCL of *E. coli* TOP10 cells transformed with pBAD-Thio-TbpB-V5-His induced with 0.075% arabinose, 37 °C, 2 h (optimal induction conditions); lane 2, WCL of *E. coli* TOP10 cells transformed with pBAD-Thio-V5-His induced with 0.075% arabinose, 37 °C, 2 h (positive control); lane 3, WCL of *E. coli* TOP10 cells induced with 0.075% arabinose, 37 °C, 2 h (negative control). Lane X, molecular weight marker at both sides of the gel.

using nickel bound to sepharose beads. The eluted fraction of the nickel affinity column was subjected to electrophoresis, then transferred to nitrocellulose membrane and the band corresponding to the TbpB-His fusion protein was cut and used for immunization and preparation of mAbs and pAbs.

3.5. Haemophilus parasuis *ExbB, TbpB and TbpA proteins are upregulated under iron-restricted conditions*

To determine the presence and regulation of expression of *H. parasuis* ExbB, TbpB and TbpA proteins, pAbs against TbpB produced during this work and against TbpA and ExbB provided by Dr. Professor Gerlach were used to identify *H. parasuis* transferrin-binding receptors. To achieve conditions of restricted iron availability, 2.2 dipyridyl (final concentration 100 μM) was added to the culture medium. *H. parasuis* and *A. pleuropneumoniae* WCL were run in SDS–PAGE and transferred onto nitrocellulose membranes and the level of protein expression determined using rabbit pAbs generated against the purified *H. parasuis* TbpB-His protein and *A. pleuropneumoniae* ExbB and TbpA fusion proteins.

The data demonstrated that *H. parasuis* ExbB protein was present in all the 15 *H. parasuis* reference strains described so far. Western blot studies showed a band around 25 kDa in size that was present in all *H. parasuis* tested as well as in the positive control (*A. pleuropneumoniae* serotype 7). When 2.2 dipyridyl was added to the culture medium, an increase in the protein expression level of ExbB protein was observed in all the reference strains of *H. parasuis* as well as in *A. pleuropneumoniae* (Fig. 4(a)).

To determine whether the expression of transferrin-binding proteins (TbpB and TbpA) of *H. parasuis* was regulated under limited-iron conditions, immunoblotting studies with rabbit pAbs against TbpB and TbpA fusion proteins were performed. A band of around 65 kDa of molecular weight corresponding to TbpB was clearly evidenced in all reference strains of *H. parasuis* as well as in the positive control, *A. pleuropneumoniae*. This band was upregulated under iron-restricted conditions when 2.2 dipyridil was added to the culture medium (Fig. 4(b)). Like the results obtained for the TbpB, immunoblotting studies using a specific rabbit pAb against TbpA protein revealed that under iron-replete conditions all strains of *H. parasuis* displayed a band of ≈106 kDa. Moreover, the expression level of this protein increased under iron-restricted conditions (Fig. 4(c)).

In summary, the data gathered support the concept that ExbB, TbpB, and TbpA protein expression increases under conditions of limited iron, as has been described for other members of the *Pasteurellaceae* family, such as *A. pleuropneumoniae* [17,18].

3.6. Immunodominance of the N-terminus domain of H. parasuis *TbpB protein*

Three specific mAbs for the GST-TbpB fusion protein (clones 10C9, 1C10 and 6D8) were obtained that recognized GST-TbpB transformed *E. coli* BL21 cell lysate in immunoblotting were identified and did not react with a negative control (bacterial cell lysate from emptied plasmid transformed in *E. coli* BL21 cells). Surprisingly, despite the fact these mAbs were generated against the complete TbpB used as antigen, in ELISA and immunoblotting they recognized the TbpB-His purified recombinant fusion protein that contained only the N-terminus domain of the *H. parasuis* TbpB protein (Fig. 5).

In addition, 11 hybridomas secreting mAbs against the N-terminal region of TbpB protein (clones 3A10, 3D6, 4D10, 6D4, 6F3, 7G5, 7G6, 8A1, 8B7, 8B8, and 10E6) were obtained. These mAbs specifically recognized TbpB-His recombinant fusion protein in ELISA. All of them, except 4D10, also recognized the purified TbpB-His fusion protein (Fig. 5) in immunoblotting. Interestingly, two of them (clones 6D4 and 7G6) also recognized a saline extract of *H. parasuis* in ELISA. No positive reactions were found when these mAbs were confronted with emptied plasmid transformed *E. coli* TOP10 bacterial cell lysate or purified Thio-V5-His fusion protein (Fig. 5).

4. Discussion

Few studies of the virulence factors involved in the pathogenicity of *H. parasuis* have been reported to date

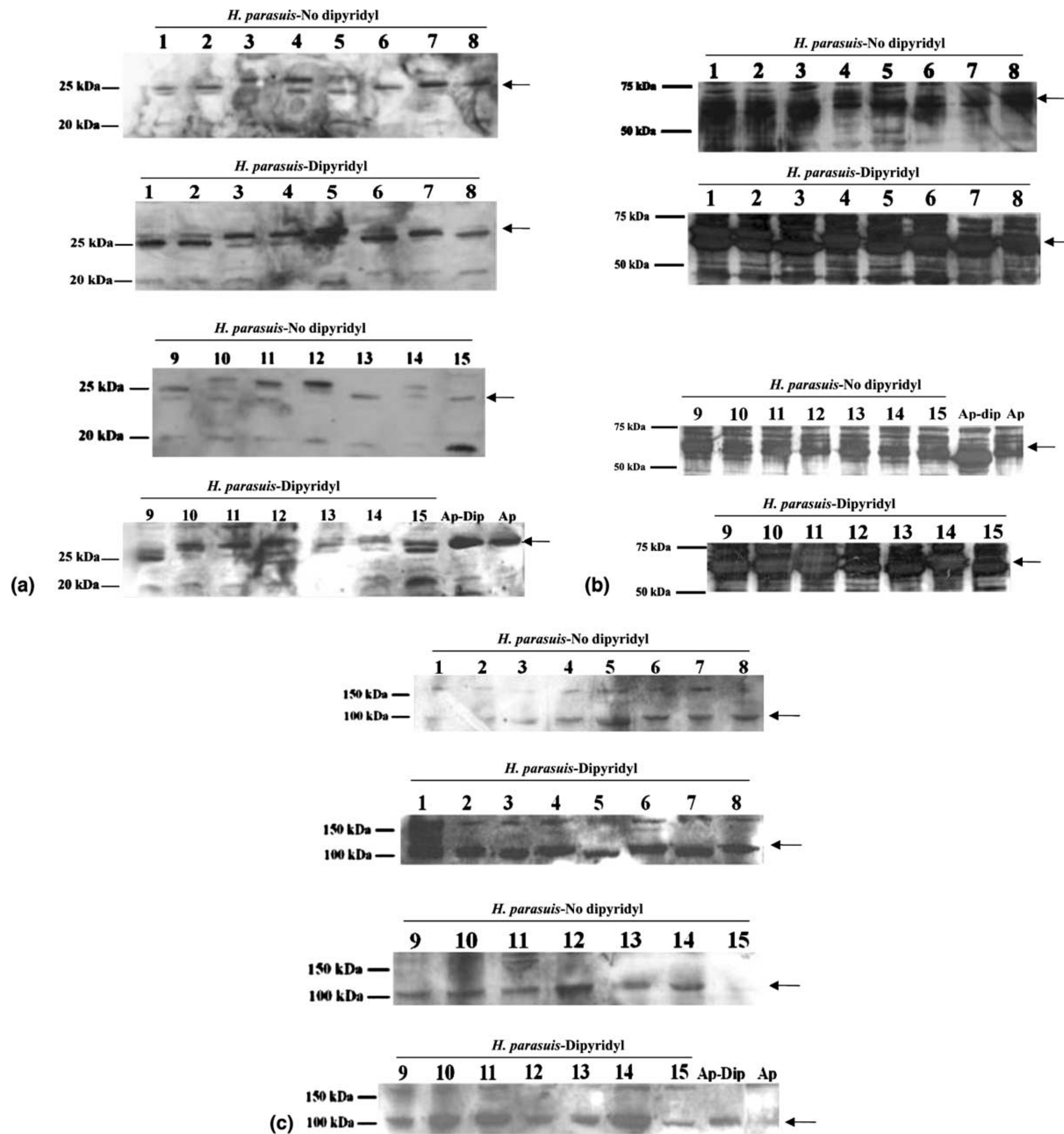

Fig. 4. (a) Immunoblotting assay using a cross-reactive pAb against *A. pleuropneumoniae* ExbB protein that recognized the homologous protein in *H. parasuis* shows ExbB expression in the 15 *H. parasuis* reference strains in the presence and absence of 2.2 dipyridyl. Top panel: *H. parasuis* serotypes 1–8 (lanes 1–8). Bottom panel: *H. parasuis* serotypes 9–15 (lanes 9–15), and *A. pleuropneumoniae* serotype 7 with (Ap-dip) and without (Ap) 2.2 dipyridyl. The protein concentration was estimated by the micro-BCA protein assay with a bovine serum albumin standard. (b) Immunoblotting using pAb against TbpB-His protein, showing TbpB expression in the 15 *H. parasuis* reference strains in presence and absence of 2.2 dipyridyl. Top panel: *H. parasuis* serotypes 1–8 (lanes 1–8). Bottom panel: *H. parasuis* serotypes 9–15 (lanes 9–15), and *A. pleuropneumoniae* serotype 7 with (Ap-dip) and without (Ap) 2.2 dipyridyl. The protein concentration was estimated by the micro-BCA protein assay with a bovine serum albumin standard. (c) Immunoblotting using a cross-reactive pAb raised against *A. pleuropneumoniae* TbpA protein, showing TbpA expression in the 15 *H. parasuis* reference strains in presence and absence of 2.2 dipyridyl. Top panel: *H. parasuis* serotypes 1–8 (lanes 1–8). Bottom panel: *H. parasuis* serotypes 9–15 (lanes 9–15), and *A. pleuropneumoniae* serotype 7 with (Ap-dip) and without (Ap) 2.2 dipyridyl. The protein concentration was estimated by the micro-BCA protein assay with a bovine serum albumin standard.

[2–8]. As in other microorganisms and owing to the well-known fact that iron availability in the host is low, the ability of the microorganisms to sequester iron is widely recognized as a virulence factor. Mechanisms for iron acquisition involve two general categories: synthesis of siderophores or receptor-mediated acquisition of iron bound to host proteins [19].

Taking advantage of the taxonomic similarities between *H. parasuis* and *A. pleuropneumoniae*, oligonucleotides for PCR were designed, based on the available

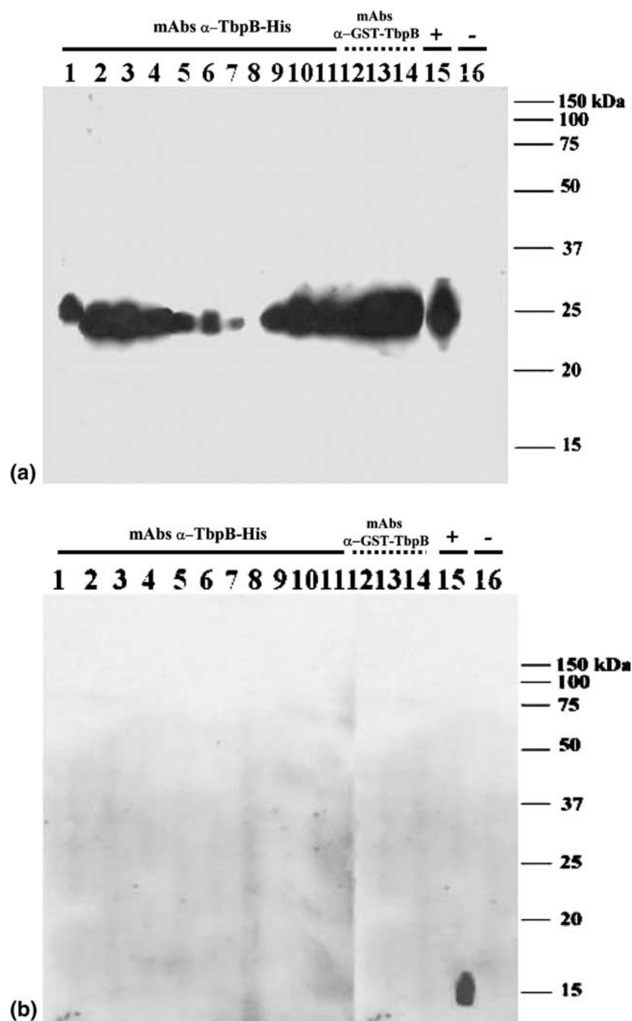

Fig. 5. Immunoblotting depicting the reactivity of the mAbs obtained against TbpB-His and against GST-TbpB fusion proteins from *H. parasuis* serotype 5. Nickel affinity chromatography purified TbpB-His protein (top panel), and negative control Thio-V5-His (bottom panel). Lanes 1–11, mAbs against TbpB-His fusion protein; lanes 12–14, mAbs obtained against GST-TbpB fusion protein; lane 15, anti-V5 mAb (Invitrogen, positive control), lane 16, mAb mouse anti-human CD3, clon OKT3 (negative control). Lane 1, mAb 10E6; lane 2, mAb 8A1; lane 3, mAb 7G5; lane 4, mAb 7G6; lane 5, mAb 3D6; lane 6, mAb 8B8; lane 7, mAb 6D4; lane 8, mAb 4D10; lane 9, mAb 3A10; lane 10, mAb 8B7; lane 11, mAb 6F3; lane 12, mAb 1C10; lane 13, mAb 6D8; lane 14, mAb 10C9; lane 15, anti-V5 mAb; lane 16, mAb clon OKT3.

sequences of *A. pleuropneumoniae tonB*, *exbB*, *exbD*, *tbpB* and *tbpA* genes. Thus, the homologous genes in *H. parasuis* were successfully amplified, cloned and sequenced, showing an identical size and organization to that of TonB region genes described in *A. pleuropneumoniae* [18] and *A. suis* [20], but different from that of other members of the *Pasteurellaceae* family. Whereas in *H. parasuis*, *A. pleuropneumoniae* and *A. suis* the *tonB* gene is followed by *exbB*, *exbD*, *tbpB* and *tbpA*, in *Pasteurella multocida*, *Mannheimia haemolytica*, or *Haemophilus influenzae*, *exbB*, *exbD*, and *tonB* genes are located consecutively, but not linked to *tbpB* and *tbpA* genes [21–23]. In *H. parasuis*, like *A. pleuropneumoniae* or *A. suis*, the *tonB–exbBD–tbpBA* organization could indicate that these genes were acquired simultaneously to carry out efficiently the uptake of iron bound to transferrin [24–27]. This is in agreement with the absence of consensus promoter sequences upstream of each start codon of the TonB region genes, except for the *tonB* gene, which contained a promoter sequence preceding its start codon. Interestingly, this consensus sequence was found to be identical to that described in *A. suis* [20]. These observations might indicate that the expression of these genes is likely to be under the transcriptional control of the same promoter. In contrast, in other species of the *Pasteurellaceae* family, such as *P. multocida*, *exbB*, *exbD* and *tonB* are physically linked and their transcription is independently regulated, because each of these genes is preceded by its own promoter [22].

A novel *A. pleuropneumoniae tonB* gene (termed *tonB*2) [28] has recently been reported. This gene is transcribed along with *exbB*2 and *exbD*2, but it is not joined with others genes involved in iron acquisition. Interestingly, the organization of TonB2 region was not the same as that of TonB region previously described in *A. pleuropneumoniae* [18], *H. parasuis* and *A. suis* [20], but it was identical to the TonB region found in *M. haemolytica* or *H. influenzae* [21–23]. The $tonB^{-}$ mutant of *A. pleuropneumoniae* could grow in presence of porcine hemoglobin and hemin, but not with porcine transferrin provided as the sole iron source, whereas the $tonB2^{-}$ mutant did not grow in culture medium with any of the iron source above mentioned. This indicates that TonB region genes in *H. parasuis* may play a crucial function in transferrin-mediated iron uptake.

In most bacteria, the genes controlling iron uptake are regulated by the Fur protein, which acts as a transcriptional repressor of iron-regulated promoters by virtue of its Fe^{2+}-dependent DNA binding activity. Under iron-enriched conditions Fur binds to Fe^{+2} and acquires a configuration able to bind target DNA sequences (so called, Fur boxes or iron boxes), and inhibits transcription of most of the genes and operons repressed by this metal. On the contrary, when iron availability is limited, the RNA polymerase accesses cognate promoters and genes involved in iron uptake are expressed [29]. The data presented here support the hypothesis that the upregulation of the TonB region proteins seen under iron-restricted conditions might be due to the presence of a unique promoter, preceding the TonB region and the regulatory Fur box sequence that was identified in *H. parasuis* overlapping with the −10 consensus promoter sequence. A similar upregulation of the TonB region proteins has been described in other members of *Pasteurellaceae*, such as *A. suis* [20], in which a Fur

box has been located preceding the start codon of the tonB gene.

According to the data gathered in this work, it appears that the N-terminus domain of the *H. parasuis* TbpB might represent an immunodominant region, since the antibodies raised against the whole TbpB protein turned out to recognize the N-terminus domain of that protein, as demonstrated when they were confronted with the N-terminal domain of the TbpB-His protein. On the other hand, the majority of the mAbs generated against the N-terminus domain of TbpB protein recognized the TbpB-His fusion protein in ELISA and immunoblotting. Interestingly, although the majority of hybridomas secreting mAbs anti-TbpB recognized putative linear epitopes detectable in immunoblotting, two of them (clones 6D4 and 7G6) also recognized in ELISA a saline extract of *H. parasuis*, in which the TbpB protein may be in its native state.

Several potential utilities of the mAbs could be derived from a diagnostic point of view. Ongoing experiments are being undertaken in our laboratory, in an attempt to determine whether they could be used in a sandwich ELISA format as capture antibodies for the TbpB recombinant protein and thus develop diagnostic tools for monitoring serum samples from convalescent pigs. Another possibility that is being evaluated is whether these mAbs could be used in a competition ELISA assay for the diagnosis of Glasser's disease.

Studies in *N. meningitidis* or *A. pleuropneumoniae* have demonstrated the potential efficacy of TbpB as a vaccine antigen [12,30]. The detection and production of a soluble and purified form of *H. parasuis* recombinant TbpB-His, that is likely to be surface accessible to antibodies, provides an opportunity to directly assess whether this antigen can serve as an effective vaccine antigen to protect not only against serotype specific *H. parasuis*, but also against other serotypes of *H. parasuis*. Previous experiments immunizing with recombinant TbpB from *N. meningitidis* [12] have shown that the antibodies generated against the N-terminus domain were sufficient to develop an efficient immune response against the infection by this microorganism.

To conclude, this work reports for the first time, a detailed characterization of the TonB region and its role in transferrin-mediated iron acquisition in *H. parasuis*.

Acknowledgments

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