Cloning, sequencing and expression of the transferrin-binding protein 1 gene from *Actinobacillus pleuropneumoniae*

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Two outer-membrane proteins are involved in the uptake of iron from transferrin by certain Gram-negative bacteria, transferrin-binding proteins 1 and 2. The gene encoding transferrin-binding protein 1 from a serotype 1 isolate of the Gram-negative pathogen *Actinobacillus pleuropneumoniae* was cloned, and a fragment encoding 700 amino acids of Tbp1 was expressed in *Escherichia coli*. We also report here sequencing of the *tbp1* gene and a comparison of the deduced amino acid sequence with Tbp1s from related species. The predicted polypeptide product of *tbp1* is a 106 kDa protein with a 22-residue signal peptide.

INTRODUCTION

The biological role of the outer membrane of Gram-negative bacteria requires the presence of selective pores that allow the transport of nutrients and waste products at a rapid rate [1]. These pores are constituted by outer-membrane proteins, some of them acting as high-affinity energy-dependent transport systems [2]. Such systems are multifunctional (a single receptor may serve for different ligands) and multicomponent (more than one periplasmic and cytoplasmic protein is involved in the transport system) [3]. Iron is one of the nutrients that must be obtained from the host for the growth of the pathogen. This is generally accomplished in Gram-negative bacteria by means of siderophore production and transport of the siderophore–iron complex across the outer membrane [4]. Certain Gram-negative pathogens, however, do not synthesize siderophores, and iron acquisition requires the direct binding of iron-carrying serum glycoproteins, such as transferrin and lactoferrin, to cell surface receptors [4]. Several *Actinobacillus* [5,6], *Haemophilus* [7,8], *Neisseria* [9,10], *Pasteurella* [11,12] and *Moraxella* [11] species seem to possess a mechanism consisting of two different proteins expressed under iron-limiting conditions, transferrin-binding proteins 1 (Tbp1) and 2 (Tbp2), specific for transferrin from the host [9]. A pathway for iron acquisition has been suggested [13], involving iron removal from transferrin at the bacterial surface by the co-ordinate action of Tbp1 and Tbp2 followed by transport of iron across the outer membrane via Tbp1 and binding of iron by a periplasmic binding protein. Tbp1 is a transmembrane protein that may serve as a channel for transport of iron across the outer membrane, and Tbp2 is a lipoprotein anchored to the outer membrane by its N-terminal lipid tail [11]. The genes encoding Tbp1 from *Neisseria meningitidis* [14], *Neisseria gonorrhoeae* [15] and *Haemophilus influenzae* [16] and Tbp2 from *N. meningitidis* [14], *H. influenzae* [16] and *Actinobacillus pleuropneumoniae* [17,18] have been cloned and sequenced, although the presence of Tbp1 and Tbp2 receptors has been tested in many other bacterial species [6,11,12,19–21].

Recent observations seem to attribute an important vaccine potential to Tbps from related species, thus making the Tbp family of proteins good candidates for vaccine components [22,23]. *A. pleuropneumoniae* is a Gram-negative bacterium responsible for contagious porcine pleuropneumonia, a disease that is distributed world wide and causes great losses in the pig industry [24]; no current vaccines provide effective protection. Here we report the cloning and sequencing of the *tbp1* gene encoding *A. pleuropneumoniae* Tbp1 from a serotype 1 isolate. We also show the expression in *Escherichia coli* and purification of a 700-amino acid fragment of the protein, which represents 76% of the mature protein.

EXPERIMENTAL

Bacterial strains, plasmids and media

The *A. pleuropneumoniae* serotype 1 strain used in this study was obtained from the American Type Culture Collection (A.T.C.C. no. 27088). *E. coli* XL1Blue [25] and BL21(DE3) [26] (Novagen) strains were used in all transformation experiments. The vectors used for cloning were pUC119 and pET-22b(+) [26] (Novagen). *A. pleuropneumoniae* was routinely maintained in tryptose blood agar (Difco) at 37 °C. Bacteria were grown on BHI medium (Difco) supplemented with β-NAD (20 mg/l; Sigma). *E. coli* transformants were grown in Luria broth medium (LB) [27] supplemented with ampicillin (50 μg/ml). After transformation with recombinant plasmids, cells were plated on LB plates supplemented with ampicillin (50 μg/ml) (for pET constructs) or LB/ampicillin supplemented with 5-bromo-4-chloro-3-indolyl β-D-galactoside (50 μg/ml) and isopropyl β-D-thiogalactoside (IPTG; 50 μg/ml) (for pUC119 constructs).

Preparation of DNA and Southern blotting

Genomic DNA was prepared as previously described [28]. Plasmid DNA was prepared from overnight cultures by alkaline lysis and a CsCl/ethidium bromide gradient centrifugation [27]. DNA fragments of the desired length and single restriction bands were recovered from agarose gels (0.8–1.5% according to DNA length) with the GeneClean kit (Bio101). All restriction endonu-

Abbreviations used: Tbp, transferrin-binding protein; LB, Luria broth medium; IPTG, isopropyl β-D-thiogalactoside; ORF, open reading frame; RBS, ribosome-binding sequence.

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The nucleotide sequence of *tbp1* gene and the deduced amino acid sequence have been deposited in the EMBL Nucleotide Sequence Database under the accession number Z49708.

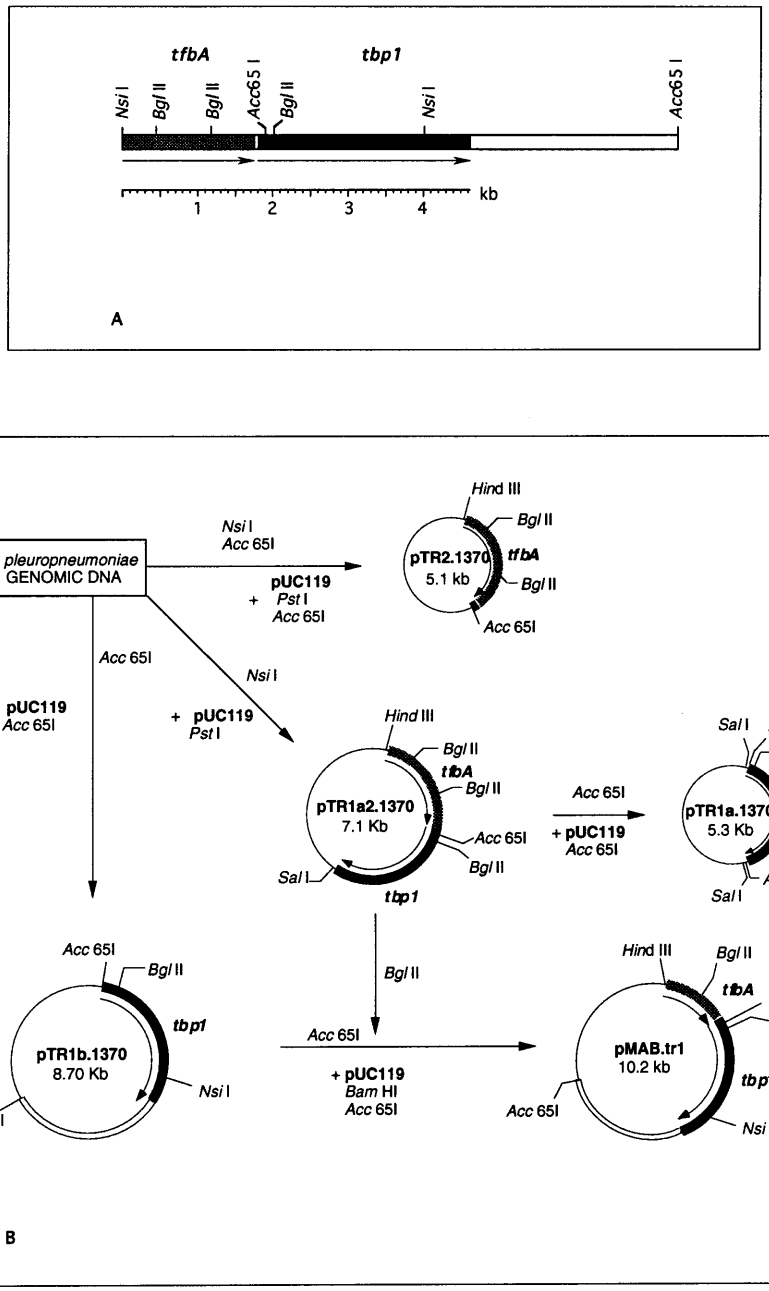

Figure 1 Physical map of the *tfbA* and *tbp1* genes in the *A. pleuropneumoniae* genome (A) and of plasmids PTR2.1370, pTR1a2.1370, pTR1a.1370, pTR1b.1370 and pMAB.tr1 used for *tbp1* sequencing (B)

In (**A**) sites for restriction enzymes used to generate the cloned fragments are indicated. Grey bars represent *tfbA*, black bars *tpb1* and white bars indicate *A. pleuropneumoniae* genome sequences flanking both genes. Thin lines in plasmids (**B**) correspond to pUC119. Sites used for the screenings are indicated.

clease digests were performed according to the manufacturer's specifications (Boehringer-Mannheim). Digested genomic DNA was separated on 0.8 % agarose gels and transferred overnight to nylon membranes (Schleicher & Schuell) by capillary blotting. DNA was cross-linked to the membrane by UV irradiation for 3 min. Probes were labelled with [α-^{32}P]dCTP (3000 Ci/mmol; Amersham) by random priming [29] with the Prime-a-gene® labelling system (Promega). Prehybridization and hybridization were performed using the Boehringer DNA labelling and detection nonradioactive kit (Boehringer-Mannheim) and the membrane was then subjected to autoradiography.

DNA sequencing

Recombinant plasmids were sequenced by using CsCl-purified or GeneClean-purified double-stranded template by the dideoxy chain termination method [30] with the T7 sequencing kit

(Pharmacia), using M13 forward and reverse primers (Clontech) or specifically designed oligonucleotides (Boehringer-Mannheim).

DNA sequence was analysed with the LaserGene package (DNASTAR Ltd.). The deduced amino acid sequence was compared with related sequences by the Clustal V method [31].

Cloning of *tbp1* for expression

pTR1a.1370 (Figure 1) was digested with *Sal*I and the insert was cloned in phase between *Sal*I and *Xho*I sites of pET-22b(+) polylinker. Genes cloned in pET are under the control of strong bacteriophage T7 transcription and translation signals, being expression induced when a source of T7 RNA polymerase is provided. As specified by the manufacturer, plasmids should initially be stabilized in a host that does not contain the T7 RNA polymerase gene, such as XL1Blue. Once established, plasmids are transferred to expression hosts, such as BL21, lysogenic for the DE3 λ phage. Clones carrying the insert in the correct orientation were identified by *Bgl*II restriction (Figure 1) and partial sequencing with oligonucleotide O_{313+} (see Figure 2).

Expression and purification of the protein

In expression experiments 2 ml from a BL21 overnight culture carrying the recombinant plasmid were used to inoculate 100 ml of LB/ampicillin and grown at 37 °C with shaking. The negative control was BL21 transformed with pET-22b(+) without insert and grown in the same conditions. Samples (1 ml) were taken 3 h after inoculation and used as controls before induction. Expression was induced at $A_{600} = 0.8$ with 0.3 mM IPTG, and 1 ml samples were taken 3 h after induction. All samples were harvested at 12000 ***g*** for 1 min and pellets were resuspended in 200 μl of 1 × SDS/PAGE sample buffer [32], boiled for 5 min and centrifuged at 12000 ***g*** for 1 min. Samples (20 μl) were analysed by SDS/PAGE (3.5 % stacking/7.5 % resolving gel) followed by Coomassie Blue staining.

pET-22b(+) vectors allow fusion of the target protein to an N-terminal sequence for periplasmic localization and C-terminal fusion to a stretch of six histidines for affinity purification of the expressed protein. The His·Tag sequence binds to Ni^{2+} immobilized on the His·Bind® metal chelation resin (Novagen). The best results in cell extract preparation were obtained under denaturing conditions. The procedure specified in the pET System Manual (Novagen) entails using 6 M urea to solubilize the protein. After 3 h of induction ($A_{600} = 2.0$), cells were harvested by centrifugation at 5000 ***g*** for 5 min. Pellets were resuspended in 40 ml of 1 × binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris/HCl, pH 7.9) and sonicated at 50 W for 20 min in an ice bath. After centrifugation at 20000 ***g*** for 15 min the pellet was resuspended in 20 ml of 1 × binding buffer and centrifuged as before. Pellets were resuspended in 5 ml of 1 × binding buffer containing 6 M urea and incubated on ice for 1 h to dissolve the protein completely. Insoluble material was removed by centrifugation at 39000 ***g*** for 20 min and supernatant was filtered through a 0.22 μm membrane before being loaded on the His·Bind® resin column for purification. After rinsing with 25 ml of 1 × binding buffer containing 6 M urea, unbound proteins were washed away with 15 ml of wash buffer (20 mM imidazole, 0.5 M NaCl, 20 mM Tris/HCl, pH 7.9, 6 M urea). Tbp1 was eluted with 1 × elute buffer (300 mM imidazole, 0.5 M NaCl, 20 mM Tris/HCl, pH 7.9, 6 M urea). The eluted protein was extensively dialysed with decreasing urea concentrations in Tris/HCl, pH 7.9, and the concentration of the protein was determined by colorimetric methods [33] using the Coomassie® Protein Assay Reagent (Pierce) and BSA as standard.

RESULTS

Cloning *tbp1* with *tfbA* as a probe

In the published Tbp2 gene (*tfbA*) of serotype 1 [17,18], an ATG initiation codon located 13 nt downstream of the end of the Tbp2 protein gene corresponds to an open reading frame (ORF) that we identified as the sequence encoding Tbp1. In serotype 1 *tfbA* is flanked by *Nsi*I and *Acc*65I sites. *A. pleuropneumoniae* serotype 1 genomic DNA was digested with both enzymes, and fragments ranging from 1800 to 2000 bp (*Nsi*I–*Acc*65I fragment comprising *tfbA* is 1902 bp long) were cloned between the *Pst*I and *Acc*65I sites of pUC119 (Figure 1). Recombinant plasmids were screened by *Bgl*II restriction, and sequencing provided further confirmation of the identity of the fragment. The plasmid containing *tfbA* was named pTR2.1370 (Figure 1).

Southern blots of *A. pleuropneumoniae* genomic DNA digested with *Nsi*I, *Bam*HI, *Hin*dIII and *Eco*RI probed with [α-^{32}P]dCTP-labelled *tfbA* allowed the identification of a single 4.1 kb band (results not shown). Cloning of 3500–4500 bp *Nsi*I fragments into the *Pst*I site of pUC119 and screening with *Bgl*II provided a clone containing *tfbA* plus 2202 nt of *tbp1* which was designated pTR1a2.1370. This clone was digested with *Acc*65I to liberate the fragment comprised of nucleotides 104–2202 of the *tbp1* coding sequence and subcloned into the *Acc*65I site of pUC119. The resulting recombinant clone was named pTR1a.1370 (Figure 1).

Sequencing of *tbp1* gene

The first 104 coding nt of *tbp1* were sequenced from the recombinant plasmid pTR2.1370 with M13 forward primer. By these means we also confirmed the sequence of the 13 nt intergenic region between *tfbA* and *tbp1* [18].

Restriction of the pTR1a.1370 insert with *Alu*I (Figure 2) yielded a set of fragments that were cloned in the *Sma*I site of pUC119. Both strands of the inserts were sequenced with M13 forward and reverse primers. Complete sequencing of the pTR1a.1370 insert was also achieved with the oligonucleotides specified in Figure 2. The insert did not comprise the entire *tbp1* sequence. Thus the recombinant plasmid was digested with *Acc*65I, [α-^{32}P]dCTP-radiolabelled and used as a probe to identify the 3′ end of the *tbp1* coding sequence. *A. pleuropneumoniae* genomic DNA digested with *Acc*65I was hybridized to the probe and a 5.6 kb band was detected. Fragments between 4.5 and 6 kb were cloned in the *Acc*65I site of pUC119. Screening was performed by *Acc*65I and *Nsi*I restriction and a positive clone containing the nt 104–2796 fragment of the *tbp1* coding sequence was designated pTR1b.1370. The stop codon of the coding sequence was reached with primers O_{1959+} and O_{2455+} (Figure 2).

Analysis of the *A. pleuropneumoniae tbp1* gene and its product

A single ORF of 2796 bp was found within the sequence (Figure 2). A 4 nt sequence (GGAA) similar to canonical ribosome-binding sequences (RBS) precedes the putative start codon. The stop codon of *tfbA* is located 13 nt upstream of the start codon of *tbp1*. An inverted repeat found downstream of the *tbp1* gene could represent a transcription terminator. Except for the short RBS-like sequence, no sequences similar to prokaryotic promoter elements were found within the 13 nt intergenic region, suggesting a possible organization with *tfbA* as a single transcriptional unit, as in the *tbpA*/*tbpB* locus of *H. influenzae* [16] or the *tbp2*/*tbp1* locus of *N. meningitidis* [14].

The product encoded by the ORF corresponds to a polypeptide of 931 amino acids with a predicted molecular mass of 106 kDa. The predicted signal peptide is 22 residues long and exhibits the

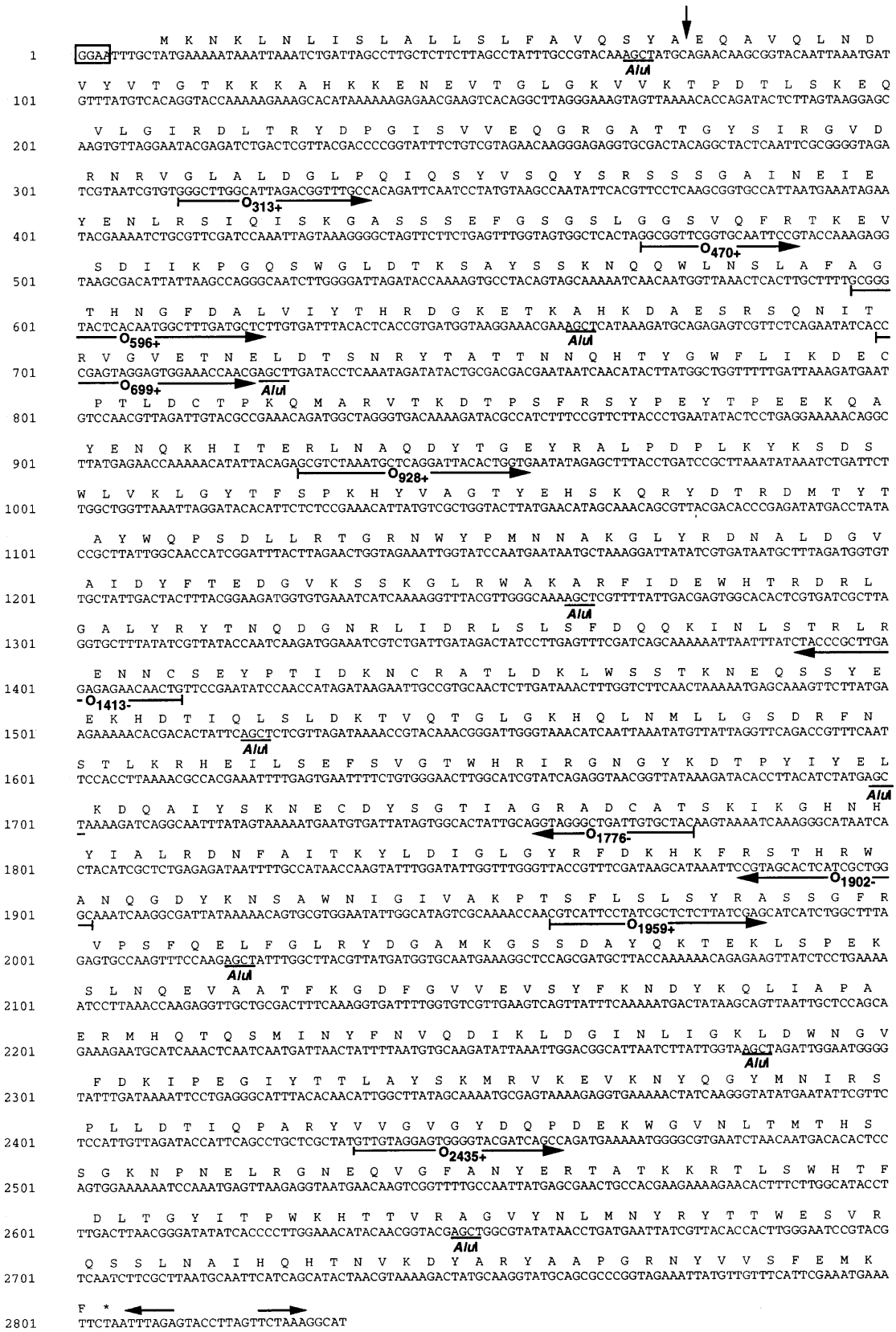

Figure 2 Complete *tbp1* sequence and translation

Deduced amino acids are positioned over the first letter of each codon. The vertical arrow indicates the predicted cleavage site of the signal peptide. Nucleotides that are boxed correspond to an RBS-like sequence. Horizontal arrows over the nucleotide sequence indicate inverted repeats that could correspond to termination sequences. The stop codon of the coding sequence is indicated by an asterisk. Arrows under the nucleotide sequence indicate the positions of oligonucleotides used as primers for the gene sequencing. Signs in the oligonucleotide names indicate the strand synthesized. Also marked are the *Alu*I sites used to generate fragments for *tbp1* sequencing.

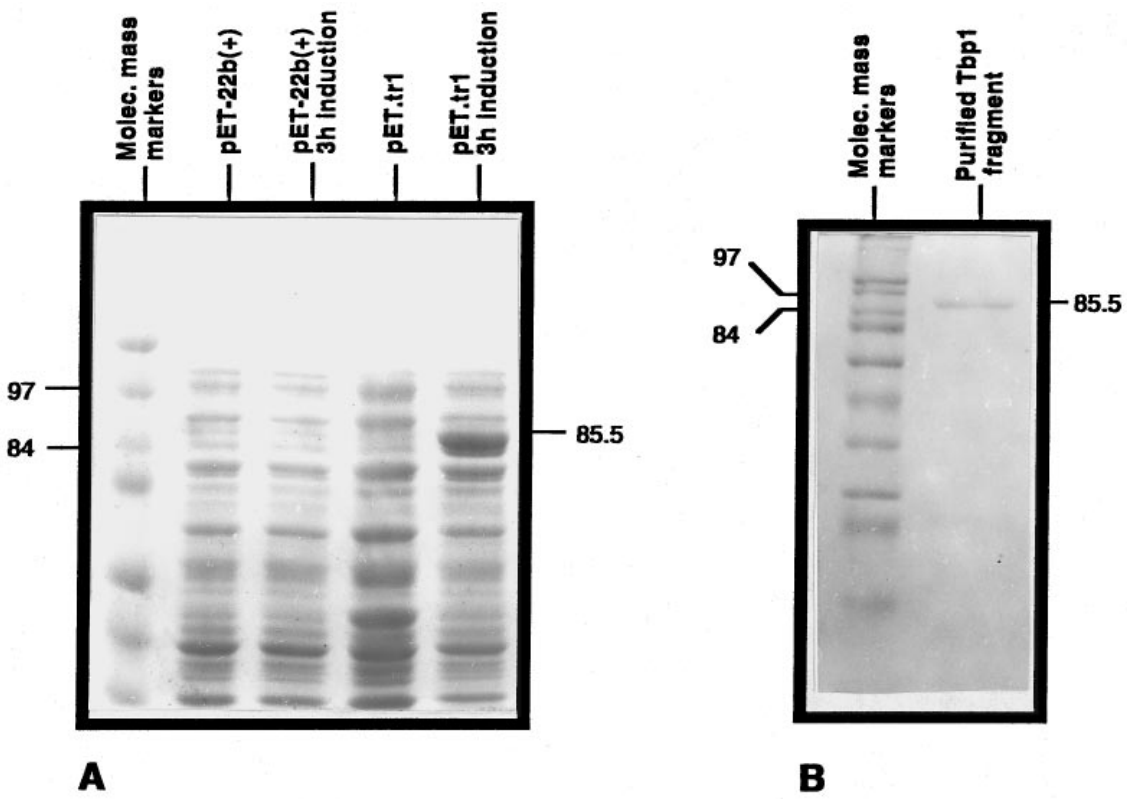

Figure 3 Expression of Tbp1 in *E. coli* and purification

(**A**) Coomassie Blue-stained SDS/polyacrylamide gel of whole cell lysates from non-induced and induced cultures of pET-22b(+) and pET.tr1. Electrophoresis was performed on 3.5% stacking/7.5% resolving SDS/polyacrylamide gel. Lanes were loaded with 20 μl of each fraction. Sigma Wide Range SDS/PAGE molecular-mass standards (5 μl) were run for comparison. Numbers on the left indicate the molecular mass in kDa of specific marker bands. Induction of pET.tr1 produces an 85.5 kDa polypeptide concordant with the molecular mass expected for the expression product. (**B**) Coomassie Blue-stained 3.5% stacking/12% resolving SDS/polyacrylamide gel shaving the purified fusion product. Numbers on the left indicate the molecular mass of specific marker bands.

features of a typical signal peptide [34]. The predicted first residue of the mature protein is the negatively charged glutamic acid at position 23, thus yielding a mature protein of 104 kDa. The calculated pI of the protein is 9.2, making it a very basic protein. The last residue of the mature protein is a phenylalanine, as in most other bacterial outer-membrane proteins [35], which seems to be essential for the correct assembly of the bacterial outer-membrane protein. Other common features are hydrophobic residues in positions 3, 5, 7 and 9 from the C-terminus. These similarities are found in outer-membrane proteins with completely different functions, suggesting an important role in outer-membrane localization, maybe in association with peptidoglycans [35,36].

Tbp1 expression and purification

Cloning of the pTR1a.1370 insert in pET-22b(+) and transformation of *E. coli* BL21(DE3) resulted in a number of clones that were screened by *Bgl*II restriction. One of the positive clones obtained was used for the expression assays and was designated pET.tr1. The selected clone contained the *tbp1* fragment encoding the 700-amino acid polypeptide Gly^{35}–His^{734}. The induced BL21 cultures carrying pET.tr1 expressed a polypeptide product that was absent from non-induced cultures or induced cultures of BL21 carrying pET-22b(+) without the insert (Figure 3A). This polypeptide migrated in concordance with the molecular mass expected for the fusion product (85.5 kDa). Proteins extracted under denaturing conditions were loaded on a His·Bind® metal chelation resin column and washed with 20 mM imidazole. The bound protein was eluted with 300 mM imidazole. Fractions containing the Tbp1 fragment were pooled and extensively dialysed with 20 mM Tris/HCl, pH 7.9, containing decreasing amounts of urea. The final dialysis buffer contained 1 M urea. At 0.5 M urea the protein precipitated irreversibly. Colorimetric quantification of the protein with BSA as standard revealed a recovery of 0.6 mg/100 ml of culture. SDS/PAGE analysis of the purified product revealed a single band that corresponds to the 700-amino acid Gly^{35}–His^{734} fragment (Figure 3B). Iron-saturated transferrin (The Binding Site Ltd.) coupled to biotin (Boehringer-Mannheim Biotinylation Kit) as described by Schryvers [8] failed to detect the 700-amino acid Tbp1 fragment, either dot-blotted or Western-blotted on to nitrocellulose membranes (results not shown). This may suggest that denatured Tbp1 cannot be recognized by porcine transferrin, as previously reported [6].

Analysis of similarity to Tbp1 from related species

A search of the EMBL and GenBank databases for homologies with the Tbp1 predicted sequence identified other Tbp1s. Figure 4 shows a Clustal-generated alignment of Tbp1 from *A. pleuropneumoniae*, *N. meningiditis* and *H. influenzae*. The overall simi-

Figure 4 Alignment of Tbp1 sequences from *A. pleuropneumoniae*, *N. meningitidis* and *H. influenzae*

The Clustal V-generated alignments of Tbp1 sequences from *A. pleuropneumoniae* (Ap), *N. meningitidis* (Nm) and *H. influenzae* (Hi) yield a significant overall similarity degree: about 37% between *A. pleuropneumoniae* Tbp1 and the other Tbp1s compared.

larities between *A. pleuropneumoniae* Tbp1 and the other proteins are 37.1 % for *H. influenzae* Tbp1 and 36.8 % for *N. meningitidis* Tbp1. However, there are specific domains that show a much higher similarity, particularly within the N-terminal and C-terminal regions of the mature protein. There are stretches of 34 amino acids (for instance between Ile^{68} and Val^{101}) that exhibit 100 % similarities at least in two of the sequences reported.

DISCUSSION

Porcine pleuropneumonia is a widespread disease with no effective protection currently available. Recent interest has been focused on virulence factors shared by all serotypes, such as outer-membrane proteins [37–39]. The expression of the 700-amino acid fragment of *A. pleuropneumoniae* Tbp1 presented here could be the first step to testing the immunogenic capacity of the protein. The fragment expressed represents 76 % of the mature protein, which may contain most of the specific epitopes of the receptor. The fact that in this expression system the protein is produced in an insoluble form represents an obstacle for functional assays such as transferrin binding and antiserum binding, although it may not be a problem for immunogenic assays, as aggregated insoluble antigens may potentiate the immune response.

In this work we have also sequenced the *tbp1* gene from *A. pleuropneumoniae*. Cloning and sequencing of genes encoding Tbp1 and Tbp2 from *N. meningitidis* [14], *N. gonorrhoeae* [15] and *H. influenzae* [16] and Tbp2 from *A. pleuropneumoniae* [18] have provided some insights into how certain Gram-negative pathogens in which siderophores have not been so far detected may obtain iron from the host's transferrin. Analysis of the genes encoding Tbp2 and Tbp1 in *N. meningitidis*, *N. gonorrhoeae* and *H. influenzae* suggests that the genes are expressed as a single transcriptional unit [16]. Schryvers [13] proposed an iron-acquisition pathway involving binding and iron removal from transferrin at the bacterial surface by the co-ordinate action of Tbp1 and Tbp2, followed by transport of iron across the outer membrane via Tbp1 and binding of iron by a periplasmic protein, such as the TonB protein in *E. coli* [3]. Although TonB was first described in *E. coli*, a *tonB* gene has already been cloned in *H. influenzae* [40] and, as previously reported [15], Tbp1 from *N. gonorrhoeae* exhibits great similarities to TonB-dependent outer-membrane receptors from *E. coli* and *Pseudomonas putida*. This affirmation may also be valid for *A. pleuropneumoniae* Tbp1, as there are many common features exhibited by the two sequences (Figure 4). Some of these similar domains have been suggested to be involved in TonB interaction and outer-membrane association. In *E. coli* the TonB protein is anchored in the cytoplasmic membrane and contacts ligand-specific outer-membrane receptor proteins involved in energy-dependent transport processes. One of the best-known proteins of this group is FepA, an outer-membrane receptor for the *E. coli* siderophore enterobactin [3]. A widespread property of most outer-membrane proteins seems to be the predominance of β-structure, with a channel formed by their amphiphilic β-strands arranged in a β-barrel [1,41,42]. In FepA the channel is closed at the cell surface by loops of hydrophilic peptides that selectively bind ligands [3]. The extensive sequence homology among the TonB-dependent receptors of *E. coli* and other Gram-negative bacteria [15] may indicate a common mechanism of function for all ligand-specific outer-membrane transport proteins. A common structural pattern for what has been called the 'TonB-dependent family of receptors' has been suggested.

In *A. pleuropneumoniae*, the close association between *tfbA* and *tbp1* and the lack of any promoter-like sequences within the intergenic region agrees with what has been described for *Neisseria* [14] and *Haemophilus* [16]. Analysis of the putative promoter region of *tbp2* from *N. meningitidis* [43] identified a region with significant similarity to the *E. coli* Fur-binding site. In *E. coli* and other bacteria, the *fur*-encoded repressor protein (Fur) binds specifically to promoter sequences [44,45] when iron levels in the environment are high, thus blocking the transcription of the iron-regulated genes. The *fur* gene from *N. meningitidis* has been cloned and sequenced and its predicted 16 kDa polypeptide product shows significant similarity to all known Fur sequences [43]. A comparison between the consensus Fur-binding sequence, the putative promoter regions of *tbp2* gene from *N. meningitidis* [14], *H. influenzae* [16] and the sequence upstream of the *tfbA* gene from *A. pleuropneumoniae* [18] yields a significant similarity. This observation suggests that a *fur*-encoded protein may also act in *A. pleuropneumoniae* as a repressor in the transcription of the *tfbA* and *tbp1* genes. Identification of the *fur* gene and its product would therefore be of great interest.

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