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Molecular Cloning and Characterization of A Unique 60 kDa/72 kDa Antigen Gene Encoding Enzyme I of the Phosphoenolpyruvate: Sugar Phosphotransferase System (PTS) of *Mycoplasma hyopneumoniae*[1]

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The recombinant clone expressing a 60 kDa (P60) antigen was isolated from *Escherichia coli* by screening a lambda EMBL3 genomic library using rabbit produced antiserum against *Mycoplasma hyopneumoniae*. Sequence analysis revealed that an interrupted (by a UGA codon) open reading frame coding for a 72 kDa protein (P72) may contain the P60 antigen gene. Western blot analysis with an anti-P60 monospecific antibody confirmed the presence of a P72 antigen from the total protein of *M. hyopneumoniae*, and a 72 kDa protein was also expressed in *E. coli* after changing the codon (UGA to UGG) by site-directed mutagenesis. BLAST (Basic Local Alignment Search Tool) comparison showed that the amino acid sequences of P72 share approximately 70% homology with the phosphotransferase enzyme I (PTSI) of bacteria and other mycoplasma species. The biological function of the P72 cytosolic protein was further confirmed by complementation using an *E. coli ptsI* mutant. The bacterial phosphoenolpyruvate-sugar phosphotransferase system (PTS) is known to mediate the uptake and phosphorylation of carbohydrates and to be involved in signal transduction. The immune responses of specific pathogen free (SPF) pigs and farm animals toward this unique antigen were observed. The transcription start positions of the PTSI gene were determined in *M. hyopneumoniae* and *E. coli* by primer extension experiments and the promoter site was also predicted.

Key words : glucose transport, *Mycoplasma hyopneumoniae*, phosphotransferase enzyme I.

Mycoplasma hyopneumoniae is the etiologic agent of swine enzootic pneumoniae, a chronic nonfatal disease affecting pigs of all ages. Understanding of the pathogenicity as well as efforts to control the disease by vaccination remain preliminary (*1*, *2*). In our previous work, we attempted to isolate the surface antigen genes to gain insights about the gene structure and its regulation, with the ultimate goal of developing recombinant vaccines. By screening the lambda EMBL3 genomic library using a rabbit-produced antiserum against *M. hyopneumoniae*, five clones encoding 10, 36, 38, 42, and 60 kDa antigen genes were isolated (*3*, *4*). Among them, the 42 kDa antigen was demonstrated to be part of a 65 kDa heat shock protein appearing on the membrane of *M. hyopneumoniae* (*4*).

In this report, the recombinant clone expressing the 60 kDa antigen has been further studied. By subcloning and Western blot analysis, the entire 4.6 kb *Eco*RI fragment was sequenced and analyzed. The biological function was investigated through sequence comparison, site-directed mutagenesis and complementation experiments using *Escherichia coli ptsI* mutant (*5*). The possible promoter region and ribosomal binding site (RBS) were predicted and the transcription start positions were determined by primer extension experiments. The immune responses of farm animals and specific pathogen free (SPF) pigs toward this unique antigen were also investigated.

MATERIALS AND METHODS

M. hyopneumoniae strain 232 (passage 27) was provided by Dr. R.F. Ross, Iowa State University, USA). Rabbit anti-*M. hyopneumoniae* hyperimmune serum was prepared as described previously for immunoscreening (*3*). *M. hyopneumoniae* was cultured in Friis medium (*6*) supplemented with 20% (v/v) porcine serum to stationary phase, concentrated, and used for chromosomal DNA preparation and Western blot analysis. The *E. coli* strains and plasmids used in the study are listed in Table I. Chemicals, restriction enzymes and modification enzymes were obtained mainly from Sigma (St. Louis, Missouri, USA), Boehringer-Mannheim (Mannheim, Germany) and Stratagene (La Jolla, CA, USA). The porcine sera from farm animals

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TABLE I. **Bacterial strains and plasmids used in this study.**

Strains or plasmids	Characteristics	Reference
DH5α	*lacZ*ΔM15 *endA*1 *recA*1 *hsdR*17 ($r_k^-m_k$)*deoR supE*44 *relA*1	*22*
HB101	F^-, *hsdS*20($r_B^-m_B^-$)*recA*13 *ara*-14 *proA*2 *lacY*1 *galK*2 *rpsL*20(Sm^R)*xyl*-5 *mtl*-1 *supE*44	*17*
JTL2	*ptsI*$^-$, derivative of HB101	*5, 18*
P2392	P2 lysogen of LE392 (*supE*44 *supF*58 *hsdR*514 *galK*2 *galT*22 *metB*1 *rpR*55 *lacY*1)	*10*
pUC18	*lac*$^+$$Ap^R$	*19*
pGEM-7Zf	*lac*$^+$$Ap^R$ f1 ori	US patent 4766072
pTL15	pUC18::1.5 kb[S-E]	This work
pTL46	pUC18::4.6 kb[E-E]	This work
pTL65	pUC18::6.5 kb[E-S]	This work
pTLC46	pGEM-7Zf::4.6 kb[E-E]	This work
pTLC46M	pGEM-7Zf::4.6 kb[E-E] mutation	This work

Abbreviations: E, *Eco*RI; S, *Sal*I.

(which had been immunized with traditional vaccines against *M. hyopneumoniae*) and the preimmune serum of SPF pig were generously provided by Dr. J.N. Ong of the Taiwan Pig Research Institute.

Immunoscreening and Western Blot Analysis of Recombinant Clones—M. hyopneumoniae chromosomal DNA was prepared, assembled into the lambda EMBL3 vector and *in vitro* packaged to obtain the genomic library described previously (*7–9*). Immunoscreening was performed by plating the library with *E. coli* strain P2392 (*10*). Phage plaques were lifted with nitrocellulose (NC) paper, washed with TBST (150 mM NaCl, 50 mM Tris-HCl, 0.05% Tween20, pH 8.1) and blocked with 5% skim milk in TBS (150 mM NaCl, 50 mM Tris-HCl, pH 8.1). Recombinant clones were screened with the preabsorbed rabbit anti-*M. hyopneumoniae* serum (*3*). Positive clones were then lifted from the plate and further purified to homogeneity. The size of each of the protein antigens recognized by the antiserum was determined by Western blot analysis. Total proteins from each potentially positive plaque were prepared by the mini lambda-lysate method in which the proteins on a confluent plate are collected in 3 ml SM buffer (100 mM NaCl, 16.6 mM $MgSO_4$, 50 mM Tris-HCl, pH 7.5, 0.01 M gelatin), precipitated by the addition of 1/10 volume of 100% trichloroacetic acid, and then dissolved in 80 μl 2× sample buffer (0.2 M dithiothreitol, 4% sodium dodecyl sulfate, 0.1 M Tris-HCl, pH 6.8, 20% glycerol, 0.2% bromophenol blue and the pH was adjusted to 8.0 with 1 M Tris-base, pH 9.5). The samples were analayzed by 12% SDS-PAGE. After SDS-PAGE and blotting onto NC paper (with semi-dry blotter), the protein bands were visualized by an immunoscreening procedure similar to that described above. Similar Western blot analyses using porcine sera from various sources were also performed to detect antibodies against the P72 antigen of *M. hyopneumoniae*.

Antibodies specifically against P60 were purified from the antiserum using the antigen-specific antibodies preparation method (*11*). In brief, antigens were separated by electrophoresis, transferred to NC paper, and incubated with rabbit antiserum against *M. hyopneumoniae*. The antibodies against the specific proteins were then eluted.

DNA Sequencing and Sequence Analysis—The DNA fragment containing the gene of interest was subcloned into pGEM-7Zf vector for *Exo*III directed deletion subcloning (*12, 13*). Overlapping clones were collected after size determination by 1% agarose gel electrophoresis. Single-stranded DNA sequencing was performed by the dideoxy nucleotide chain-termination method (*14*) and the sequence was aligned using the GeneWorks programs (IntelliGenetics, Mt. View, CA, USA). DNA and amino acid sequence comparisons were also performed using the BLAST program provided by the National Center for Biotechnology Information (NCBI), USA.

Mapping of mRNA 5′-Termini by a Primer-Extension Experiment—Primers (200 ng) with the same sequence as the transcribed strand, 50–100 nt downstream from the possible transcription start position (tsp) were synthesized and end labeled with [γ-^{32}P]ATP (>5,000 μCi/mmol, Amersham Life Science, Cleveland, Ohio, USA) and T4 polynucleotide kinase and purified from unincorporated isotope by Bio-Gel P-10 (50–100 meshes) filtration (*15*). Total RNAs were isolated from *E. coli* harboring plasmid pTLC46, and from *M. hyopneumoniae* using an RNA/DNA Isolation Kit (Maxim Biotech) and the hot phenol method (*15*). The primer used for the present studies was 5′-TCCGCTTGAGGCCCCGATTCC-3′, which is identical to nt 30 to nt 51 of the transcribed strand of ORF1. The annealing mixtures contained 4 μg cellular RNA and 2 pmol labelled primer in 10 μl of reverse transcription buffer (5 mM Tris-HCl, pH 8.0, 40 mM KCl, 5 mM $MgCl_2$, and 2 mM dithiothreitol). Primers were extended by the addition of 1 μl of 2.5 mM dNTP and 200 units of M-MuLV reverse transcriptase. The extended molecules were electrophoresed in a sequencing gel, in parallel with the extended DNA containing the 1.5 kb *Sal*I–*Hin*dIII fragment, using the same primer.

Site-Directed Mutagenesis—Site-directed mutagenesis of the P60/P72 gene was carried out using a QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The primers used for the PCR mutation reaction were: primer 1: 5′-CGAAAGGAAAATGGGTGGGAATG-3′ and primer 2: 5′-CATTCCCACCCATTTTCCTTTCG-3′. After PCR, the DNA was digested with *Dpn*I before transformation and screening. The mutation was further confirmed by Western blot analysis and DNA sequencing.

Protein Fractionation—The mycoplasma total proteins were fractionated using detergent Triton X-114 (*16*). The membrane proteins and soluble cytosolic proteins were then analyzed by Western blot analysis.

In Vivo Complementation Assay—Plasmids pTLC 46 and pTLC46M carrying the P60 gene and P72 gene, as well as the vector pGEM-7Zf were transformed to *E. coli* HB101 (*17*) and the *ptsI* mutant strain JLT2 (*5, 18*). Complementation was observed on MacConkey agar containing 1% glucose, revealing a purple red color change.

RESULTS

Molecular Cloning and DNA Sequencing of the Recombinant Clone Expressing P60 Antigen in E. coli—The *M. hyopneumoniae* genomic library was constructed using the lambda EMBL3 vector and screened with purified rabbit anti-mycoplasma antiserum (*3*). Recombinant clones expressing antigen proteins with molecular mosses of 60, 42, 38, 36, and 10 kDa in *E. coli* were identified (*4*). The phage clone expressing the P60 antigen contained a 12.6 kb *Sal*I

insert. Through simple restriction mapping (Fig. 1), the 1.5 kb *Sal*I–*Eco*RI, 4.6 kb *Eco*RI–*Eco*RI and the 6.5 kb *Eco*RI–*Sal*I fragments were subcloned into pUC18 (*19*), and plasmids pTL15, pTL46, and pTL65, respectively, were obtained. Western blot analysis revealed that the plasmid pTL46 can still express the P60 protein and therefore the 4.6 kb *Eco*RI fragment was subcloned to phagemid vector pGEM-7Zf to obtain plasmid pTLC46 for DNA sequencing. Overlapping clones were generated using the *Exo*III unidirectional deletion method (*12, 13*) and both strands of the 4.6 kb *Eco*RI–*Eco*RI DNA were sequenced using the dideoxy chain-termination method (*14*). As shown in Fig. 2, the 4.6 kb *Eco*RI–*Eco*RI fragment actually has 4,673 nucleotides. The G+C content of the 4.67 kb DNA is only 28.9%, typical of the low G+C content in Mycoplasma genomes (*20*). The 4.6 kb DNA sequence has been annotated in the GenBank (NCBI) with the accession number AF140575. Using the mycoplasma codons, three open reading frames: ORF1 (from nt 676 to nt 2412, 578 aa), ORF2 (nt 2586–3326, 246 aa) and ORF3 (nt 3326–4051, 242 aa) were identified in the 4.6 kb DNA (Figs. 1 and 2). It is possible that ORF1 encodes a P72 protein based on the calculated molecular weight. However, a unique TGA codon (nt 2229–2231) was observed in ORF 1 and this universal termination codon may cause a premature termination and generate a P60 protein in *E. coli*. Since many mycoplasma genes have been reported to use UGA as a tryptophan codon (*21*), the TGA may represent a tryptophan instead of a stop codon in

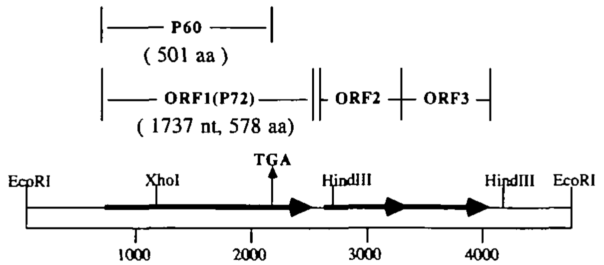

Fig. 1. **Restriction map and open reading frames (ORF) of the 4.6 kb *Eco*RI–*Eco*RI DNA fragment containing the P60 protein gene.** Possible ORFs are indicated by bold arrows.

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GAATTCAAATTTTAACGGAATTACTTTTATTTTAGGTTATTATAATTCGATTTTGTGTAAAATTTTTAGTTCCACATCTGGACTTGTAGGGAAAAAAGTC  100
TTTGGAATTGGCACTATTTTAGAAAATATTTATTTTAATATGCTTTTAAAAAAAACAAATTCTGATCTAGCAAAAACTGCTAATCTTCTTATCCTTGGAG  200
ATCAAGGAAATAGTTTTTTAATCAATCAAGATTTTAAAAATAATGAAATAACAGATAAAAATAATGAAATATTTGATAAACTTATTCAGAAAATAAATGA  300
AAAAAGTTTAGAAAAAATTATTAGAAAAACGGCAGAAAATTGACTGTTAGCATCAATTTTACTAGATATTTTACTAGATATTTTTCAGCAAAAACAAAGT  400
TTTTTCCTAATAAATTCTTGAGTTGAATCATTTTATAATATAAAAAATGATTTTTTCACAATTCCTGTCAAAATCGATCTATCAGGTCAAATTGAAACTA  500
AAAATATTAACCTAAATAAGCAGGATCAGCAAAAATTAATTCAATTTATGTGAAAAAAAGATGAACTAAACACGCTTATTGACAAATATTTATTAAAATA  600
AGATAAAAATTTTATTATTTTTTGATATAATTTTTTTTAGTAAATAGTTTTTGTGTTTAAAACTGGAAGGAAAAGATGGCAAAACTTAATTCTTATAAAT  700
                     -35                 -10         >     >        RBS   M  A  K  L  N  S  Y  K  F
                                                                          ORF1
TTAAAGGAATCGGGGCCTCAAGCGGAATTGCAATCGCAAAAGTTTTTAAATTAGTTGAACAAAAAATTGAAATTAGTGATCAAAAAGTTGAGGATATTAG  800
  K  G  I  G  A  S  S  G  I  A  I  A  K  V  F  K  L  V  E  Q  K  I  E  I  S  D  Q  K  V  E  D  I  S
CACTGAAATTCAAATTTTTGAAGAGGGGGTAAAAAAATCTGTTAGCCAAATTGAAAAAATTAAAAAATTAGCAACTAAGTTAGCAAAAGATGAGCTCGAA  900
 T  E  I  Q  I  F  E  E  G  V  K  K  S  V  S  Q  I  E  K  I  K  K  L  A  T  K  L  A  K  D  E  L  E
ATTCTTGATGCTCATATTTTGATTGCAACAGATCCTGTTCTTAAAGAAGATACAATCAATTTAATCAAGCAAGGATATTCAGCTGCTTATTCTTTAAAAG  1000
I  L  D  A  H  I  L  I  A  T  D  P  V  L  K  E  D  T  I  N  L  I  K  Q  G  Y  S  A  A  Y  S  L  K  E
AAACGGCAAAAAATCATACTAATTTTTTACTTGAAACCGGGGATGAATATTTAATGGCTCGAGCAACTGATATCAAAGATGCCTCACAAGAATTATTAA  1100
 T  A  K  N  H  T  N  F  L  L  E  T  G  D  E  Y  L  M  A  R  A  T  D  I  K  D  A  S  Q  R  I  I  K
AAATATTTTAAATTTGAATATTTTAGATTTAAGTGCAATCGATAAGGATGTAATAATTGTTGCTGATGATCTAAAGCCCTCAGATACCGCCCAACTAAAT  1200
 N  I  L  N  L  N  I  L  D  L  S  A  I  D  K  D  V  I  I  V  A  D  D  L  K  P  S  D  T  A  Q  L  N
TCATATGTTAAAGGTTTTGCAACTAATATTGGCTCAAAAACTTCGCATTCAGCAATCATGGCGCGAAGTTTAGAAATTCCAGCAATTTTAGGTGTTAGTG  1300
S  Y  V  K  G  F  A  T  N  I  G  S  K  T  S  H  S  A  I  M  A  R  S  L  E  I  P  A  I  L  G  V  S  D
ATATTCTTGAAAAAGTTGAAAATGATGATATTTTAGCAATTAATGGCGATCTTGGCTATGGAATTATTAACCCAAATCAAGAAGAAATTGAAGAATTTGA  1400
 I  L  E  K  V  E  N  D  D  I  L  A  I  N  G  D  L  G  Y  G  I  I  N  P  N  Q  E  E  I  E  E  F  E
GCGCCAAAAAGCCAATTTTGAAGCTGAAAAAGAAAAATTACAAGAATTTTTAAATAAAAAAGCAACTAGTGCTGATGGAAAAAAGGTTGTAATTGCTGCT  1500
 R  Q  K  A  N  F  E  A  E  K  E  K  L  Q  E  F  L  N  K  K  A  T  S  A  D  G  K  K  V  V  I  A  A
AATATTGGATCAGTTGAAGATTCATATGCAGCAAAAAAAGTAAATGCTGATGAAATTGGGCTTTTTCGCTCAGAATTTTTATATATGGACAGGTCAAATT  1600
N  I  G  S  V  E  D  S  Y  A  A  K  K  V  N  A  D  E  I  G  L  F  R  S  E  F  L  Y  M  D  R  S  N  W
GGCCTTCTGAAGAAGAGCAATTTTTAAGTTATAAAGCCGTAATCGAGAGTCAAAATCCGAAAAAAGTTGTTGTTAGAACCCTTGATATCGGCGGCGATAA  1700
 P  S  E  E  E  Q  F  L  S  Y  K  A  V  I  E  S  Q  N  P  K  K  V  V  V  R  T  L  D  I  G  G  D  K
AACTTTAAAATACTTTGATTTTGCTAAGGAATTAAACCCTTTTTTAGGCTATCGGGCAATTCGTCTTTGTCTTGATAAATTAGAAATTTTTAAAACTCAG  1800
 T  L  K  Y  F  D  F  A  K  E  L  N  P  F  L  G  Y  R  A  I  R  L  C  L  D  K  L  E  I  F  K  T  Q
CTTCGGGCACTAGTAAGGGCTTCTGAATTTGGAAATTTAGCAATTATGTTTCCCATGGTTGCAAGTCTTGATGAATTTTTTCAGGCAAGAGCGGTTTTTG  1900
L  R  A  L  V  R  A  S  E  F  G  N  L  A  I  M  F  P  M  V  A  S  L  D  E  F  F  Q  A  R  A  V  F  D
ATGAGGTATATAATCAAATTAAGGCTAAATATCCAAATGTTGCTCCAAAAAGCAACATTAAAATCGGAATTATGATTGAAATTCCGATTTCTGCAATTCT  2000
 E  V  Y  N  Q  I  K  A  K  Y  P  N  V  A  P  K  S  N  I  K  I  G  I  M  I  E  I  P  I  S  A  I  L
TGCTGATAAGTTTGCCGAACATGTTGATTTTTTCTCTATTGGCACAAATGATTTAATTCAATACTCCTTTGCCGCTGATCGAATGAATGAAAAAGTCGCT  2100
 A  D  K  F  A  E  H  V  D  F  F  S  I  G  T  N  D  L  I  Q  Y  S  F  A  A  D  R  M  N  E  K  V  A
TATTTATATCAAACTTTAAACCCCGGTCTGTTAAAACTTATTAAAATGACGATTGATGCCGCCCATGCGAAAGGAAAATGAGTGGGAATGTGTGGCGAAA  2200
Y  L  Y  Q  T  L  N  P  G  L  L  K  L  I  K  M  T  I  D  A  A  H  A  K  G  K  W  V  G  M  C  G  E  M
TGGCAGGGGATATCAATGCTGTACCGCTTTTATTAGGTCTTGGACTTGATGAATTTTCAGTTTCTGCAAGTAGTGTTTTAAAAGTTAAGAAACTAATTTC  2300
 A  G  D  I  N  A  V  P  L  L  L  G  L  G  L  D  E  F  S  V  S  A  S  S  V  L  K  V  K  K  L  I  S
CGAACTAAATTATCAAAAAATGCAGGAAATAGCAAAAAAAACGCTTGAATTTTCAACTGAAGAGCAAGTAATAAAATATCTTGAGTCTTTAAATTTAATT  2400
 E  L  N  Y  Q  K  M  Q  E  I  A  K  K  T  L  E  F  S  T  E  E  Q  V  I  K  Y  L  E  S  L  N  L  I
CGCCATAACTAAAAAAGCACTTAAATTTAAAGACTTTTTTATATTAAATACAAAAAAATATCAAAAACCGAAAAATTTAAAAAAAAATAATATAATTTTT  2500
R  H  N  stop
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Fig. 2 (continued on next page)

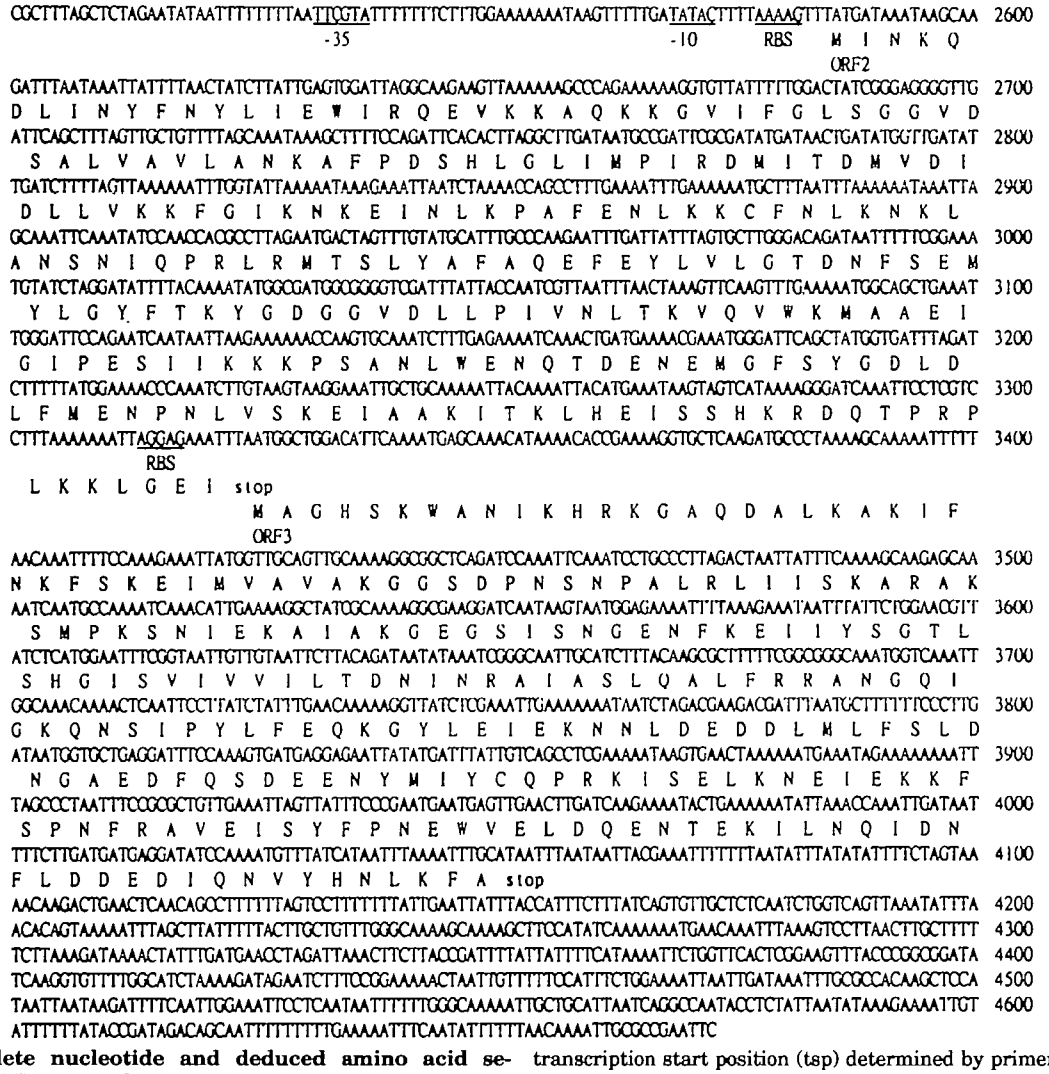

Fig. 2. **Complete nucleotide and deduced amino acid sequences of ORF1 (P72), ORF2, and ORF3. The open reading frames are indicated by bold arrows.** The possible –10, –35, and RBS for each ORF are underlined. The symbol ">" represents the transcription start position (tsp) determined by primer extension experiments. The UGA codons are represented by underlined bold letters, and the stop codons are marked by the symbol "stop."

M. hyopneumoniae. Therefore, the possibility that P60 may be part of the P72 antigen molecule was further examined.

The P60 Protein Belongs to a 72 kDa Antigen in Mycoplasma hyopneumoniae—To understand the actual size of the P60 related antigens in *M. hyopneumoniae*, monospecific antibodies against P60 protein were purified (*11*). Western blot analysis revealed that the P60-specific antibody Ab_{60} indeed reacts with a 60 kDa protein band in the total protein isolated from *E. coli* DH5α harboring pTL46 (Fig. 3). However, the Ab_{60} antibody also identified a 72 kDa protein from the total protein of *M. hyopneumoniae*. The results strongly suggested that TGA_{223} is used as a tryptophan codon in *M. hyopneumoniae* and yields a 72 kDa antigen protein. This possibility was further examined by site-directed mutagenesis. Using the QuikChange Site-Directed Mutagenesis Kit, TGA_{223} was mutated to TGG_{223}, which codes for tryptophan in *E. coli*, and pTLC46M was obtained, which is the mutated form of pTLC46. Western blot analysis using the P60-specific antibody was then performed to verify that the plasmid pTLC46M can produce a P72 protein in *E. coli*. As shown in Fig. 3, the monospecific antibody Ab_{60} can indeed recognize a P72 protein band from *E. coli* DH5α harboring pTLC46M, which is exactly the same size as appears among the total proteins of *M. hyopneumoniae*.

Transcriptional Analysis of the P72 Gene in E. coli and M. hyopneumoniae—The results of site-directed mutagenesis clearly demonstrated that ORF1 constitutes a P72 gene in *M. hyopneumoniae*. The monospecific antibody Ab_{60} reacted with a P60 protein from DH5α (*22*) harboring (pTLC46), while a P72 protein was recognized from the total protein of DH5α (pTLC46M). Through sequence comparison (*23*), a possible –35 region (5′-TTGATA-3′ and –10 region (5′-TAAATA-3′, 54–29 nt upstream from ORF1 were predicted as the potential promoter of ORF1. A sequence similar to RBS, 5′-GAAGGA-3′, 9–4 nt from ATG of ORF1 could also be identified (Fig. 2). The assignments of these regulatory sequences would require thorough mutation studies. However, a clear transcription start position would support the prediction. The transcription start position (tsp) was therefore determined by primer extension experiments as described in "MATERIALS AND METHODS." As

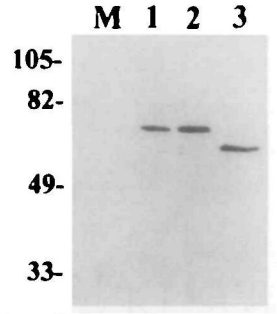

Fig. 3. **Identification of the antigen protein by site-directed mutagenesis and Western blot analysis.** The TGA_{2231} (see Fig. 1) was changed from A to G (*i.e.* plasmid PTLC46 was mutated to obtain plasmid pTLC46M) by site-directed mutagenesis, and the codon was converted to TGG codes for tryptophan in *E. coli*. The plasmids were then used to transform *E. coli* DH5α and analyzed by Western blot analysis using the monospecific antibody Ab_{60}. Lane M, 1, 2, and 3 represent protein size markers (105, 82, 49, and 33 kDa), mycoplasma total protein, DH5α (pTLC46M) total protein, and DH5α (pTLC46) total protein, respectively.

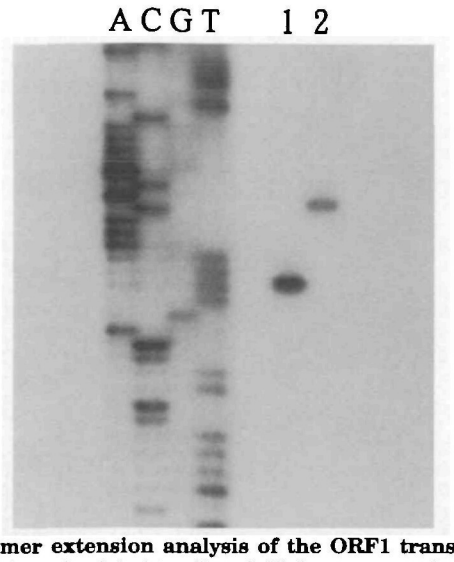

Fig. 4. **Primer extension analysis of the ORF1 transcription start positions (tsp) in *E. coli* and *M. hyopneumoniae*.** The total RNA was produced from *E. coli* and *M. hyopneumoniae*, reverse transcribed and analyzed by polyacrylamide gel electrophoresis. A sequencing ladder of plasmid pTLC46 was generated using the same primer used for primer extension. Lane 1: *E. coli* DH5α harboring pTLC46M; Lane 2: *M. hyopneumoniae*; Lane ACGT, the sequence ladder.

shown in Fig. 4 (also marked in Fig. 1), the *in vivo* transcription initiation sites are located at nt 655 (G) and nt 661 (A) in *M. hyopneumoniae* and in *E. coli*, respectively. The exact locations of tsp not only show where the mRNA synthesis starts but also facilitate the prediction of the promoter and RBS of the same gene.

Biological Function, Sequence Comparison, and Characterization of the P72 Antigen—BLAST (Basic Local Alignment Search Tool) comparison showed that the amino acid sequences of P72 share 70 and 71% similarities with the phosphotransferase enzyme I gene (PTSI) of *Mycoplasma capricolum* (*24*) and *Staphylococcus carnosus* (*5*). To further characterize the biological function of the P72 protein, plasmids pTLC 46 and pTLC46M carrying the P60 gene and P72 gene, respectively, as well as the vector pGEM-7Zf, were transformed into the *ptsI* mutant strain JLT2 (*18*) and compared with *E. coli* HB101 (*17*) carrying pGEM-7Zf. Complementation was observed on MacConkey agar containing 1% glucose. The wild type *E. coli* HB101 exhibited purple red color on MacConkey agar, while the colonies of the mutant strain JTL2 were colorless. Transformation of the JLT2 strain with plasmid pKLC46M restored the purple red color, however, plasmid pTLC46 failed to complement (data not shown). This result demonstrates that the P72 protein (but not P60) is indeed enzyme I of the phosphoenolpyruvate–sugar phosphotransferase system (PTS), and the mycoplasma enzyme can complement an *E. coli ptsI* mutant.

The amino acid sequences of PTSI proteins from *Streptococcus mutans* (*25*), *Bacillus subtilis* (*26*), *Lactococcus lactis* (*27*), *M. hyopneumoniae*, *Salmonella typhimurium* (*28*), *Escherichia coli* (*29*), and *Mycoplasma capricolum* (*24*) were aligned and compared by the GeneWorks program. The consensus sequence with a 50% cutoff value displayed the residues in the constituent sequences that show a minimum of 50% identity. Enzyme I (EI) is composed of two domains of approximately equal molecular weight (*28*), the EI-N and EI-C (the NH_2- and COOH-terminal domains, respectively). As shown in Fig. 5, the EI-Cs are more conserved than EI-Ns and all the EI-Ns contain the highly conserved active site His-189 (*28*). The nuclotide sequences and amino acid sequences of enzyme I of *S. mutans*, *B. subtilis*, *L. lactis*, *M. hyopneumoniae*, *S. typhimurium*, *E. coli*, and *M. capricolum* were also analyzed as evolutionary markers by the GeneWorks program. Phylogenetic trees based on UPGMA (Unweighted Pair Group Method with Arithmetic Mean) analysis were constructed, using (A) the DNA sequences of PTSI genes, and (B) the amino acid sequences of PTSIs. The UPGMA tree shows the calculated evolutionary relationships of the sequences in the alignments. The lengths of the horizontal lines connecting one sequence to another are proportional to the estimated genetic distance. As shown in Fig. 6A, the sequences of *E. coli* and *S. typhimurium* are closely related; the sequences of *L. lactis* and *S. mutans* are clustered and distantly related to *B. subtilis*. The sequences of *M. hyopneumoniae* and *M. capricolum* are grouped together and diverge very early from the others. Somewhat different groupings of the amino acid sequences of PTSI show that, as seen in Fig. 6B, *M. capriolum* is predicted to be more closely related to Gram positive bacteria, and together with *M. hyopneumoniae*, are deviated from other Gram negative bacteria.

To locate the P72 protein, Triton X-114 phase fractionation experiments (*16*) were performed, and each fraction was analyzed by Western blot analysis. As shown in Fig. 7, Triton X-114 phase fractionation indicated that the majority of P72 to be partitioned in the cytosolic fraction. The monospecific antibody Ab_{60} can recognize P72 antigen from the total protein and the cytosolic fraction, but not from the membrane fraction of *M. hyopneumoniae*. Analysis using the GeneWork program (prediction of prokaryotic secretory signal sequence) also revealed no signal peptide sequence in the N-terminal end of P72.

Immune Responses of Farm Animals and SPF Pig toward PTSI—The phase fractionation experiments indicate that P72 may not be a membrane surface protein. This

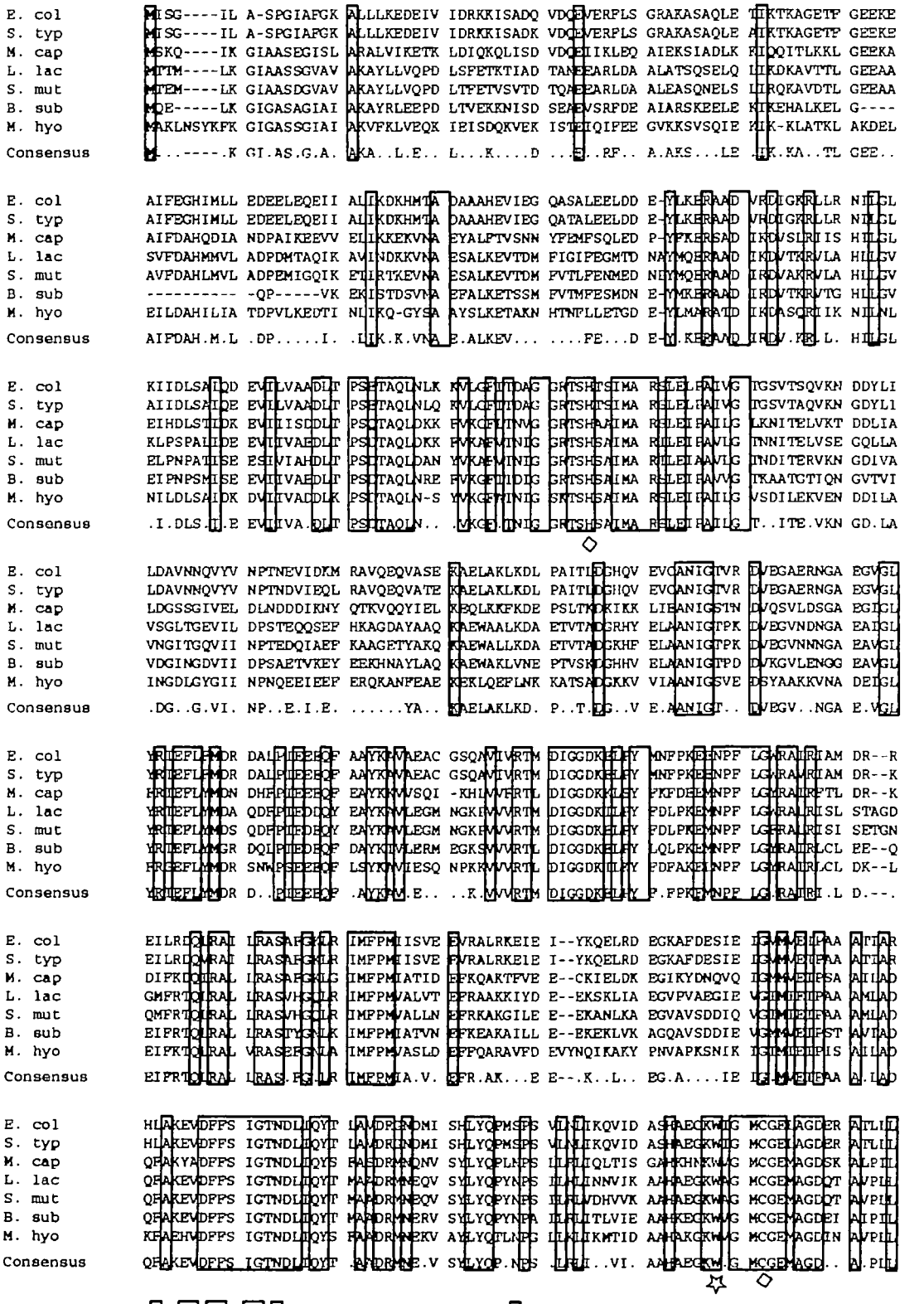

Fig. 5. **Amino acid sequence comparison of PTSI proteins.** The sequences were aligned and compared by the GeneWorks program. The boxed residues are identical. The abbreviations, S.mut, B.sub, L.lac, M.hyo, S.typ, E.col, and M.cap represent the amino acid sequences of PTSI proteins from *Streptococcus mutans, Bacillus subtilis, Lactococcus lactis, Mycoplasma hyopneumoniae, Salmonella typhimurium, Escherichia coli*, and *Mycoplasma capricolum*, respectively. The consensus sequence with a 50% cutoff value displays the residues in the constituent sequences that show a minimum of 50% identity. The star represents the Trp-502 pretermination site and the diamond symbols represent the conserved His-189 at the active site and Cys-506 required for coupling activity.

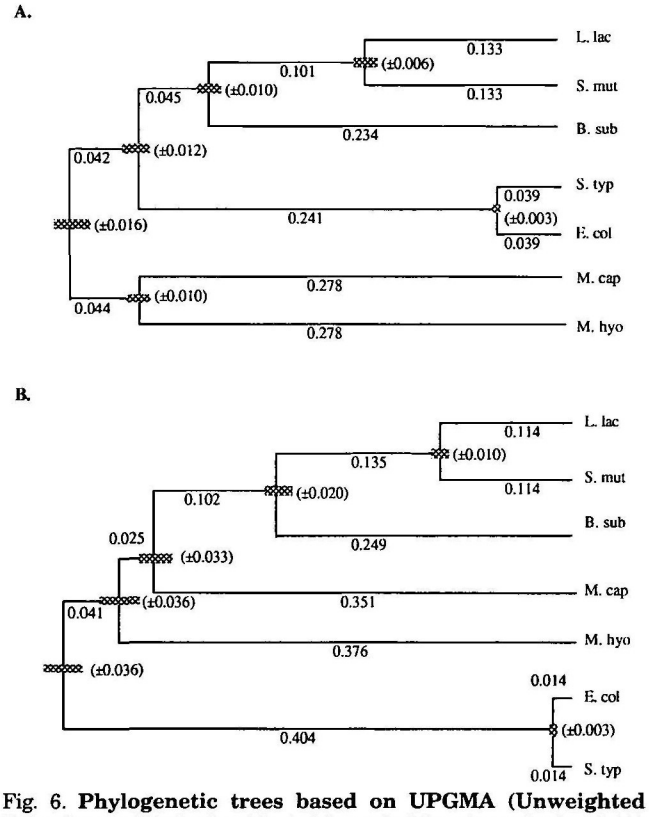

Fig. 6. **Phylogenetic trees based on UPGMA (Unweighted Pair Group Method with Arithmetic Mean) analysis of (A) the DNA sequences of PTSI genes and (B) the amino acid sequences of PTSIs.** To construct the trees, the nucleotide sequences or the amino acid sequences were aligned and compared pairwise by using the GeneWorks program to determine their identity at each position. Then, the number of mismatches was divided by the length of the shorter sequence. The results in scores (numbers on the horizontal lines of the tree) reflect the relationships between sequences. The laced lines at branch points in the tree represent standard error bars of the branch position. L.lac, S,mut, B.sub, S.tyr, E.col, M.cap, and M.hyo represent the sequences of *Lactococcus lactis, Streptococcus mutans, Bacillus subtilis, Salmonella typhimurium, Escherichia coli, Mycoplasma capricolum*, and *Mycoplasma hyopneumoniae*, respectively.

result is in agreement with the PTSI location and function of most bacterial systems. However, nonsecreted intracellular antigen was also reported to activate the host immune response (*30*). To explore this potential, porcine antisera from farm animals were screened for the presence of anti-P60/P72 antibodies. As shown in Fig. 8, the antiserum produced from an SPF pig challenged with *M. hyopneumoniae* can recognize both the P60 and P72 antigens. However,

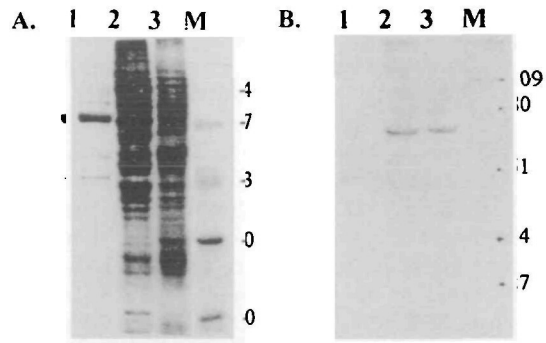

Fig. 7. **Protein fractionation of *M. hyopneumoniae* by Triton X-114 phase-partition and Western blot analysis with monospecific anti-P60 antibody.** (A) SDS-PAGE analysis of *M. hyopneumoniae* proteins. The proteins were stained with Coomassie Brilliant Blue. Lanes 1, 2, 3, and M represent membrane proteins, cytosolic proteins, total proteins, and protein size markers, respectively. (B) Western analysis. Lanes 1, 2, 3, and M represent *M. hyopneumoniae* membrane proteins (TX-114 layer), cytosolic proteins (aqueous layer), total proteins and the protein size markers, respectively.

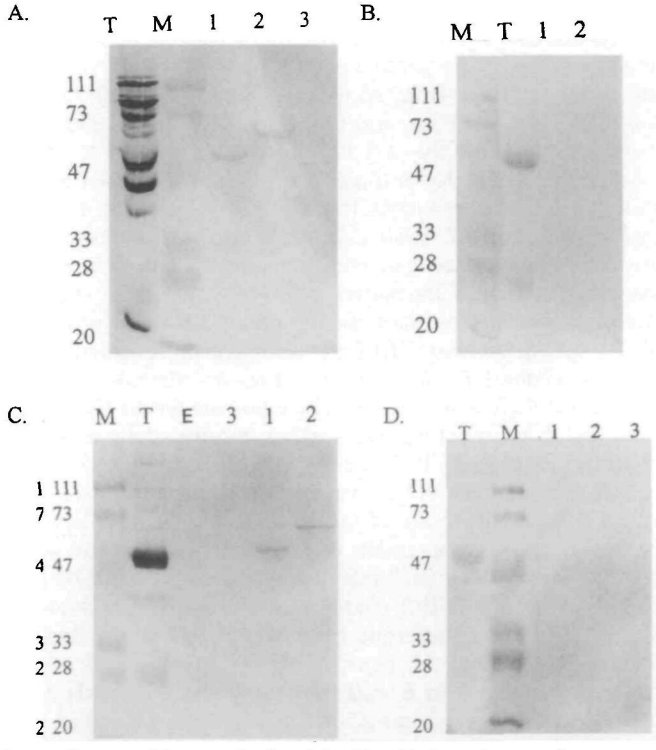

Fig. 8. **Western blot analysis of the P60/P72 antigen using various porcine antisera.** Antiserum produced from an SPF pig challenged with *M. hyopneumoniae* (A), preimmune serum from the SPF pig (B), serum D8-2 from farm animals (C), and serum D18-5 from farm animals (D) were used to check for the presence of the P60/P72 antigen. Lane T, E, 1, 2, and 3 represent the protein profiles of the total mycoplasma protein, the *E. coli* total protein, the *E. coli* strain DH5α harboring plasmid pTLC46 (expressing P60), pTLC46M (expressing P72), and pGEM-7Zf (vector), respectively. Lane M represents the prestained molecular weight standards.

only the antiserum D18-5 among the twelve tested antisera isolated from farm animals can detect the antigen. All of these farm animals had been vaccinated with a traditional

vaccine against *M. hyopneumoniae*.

The Sequence Analysis of ORF2 and ORF3—As shown in Figs. 1 and 2, three open reading frames, ORF1 (from nt 676 to nt 2412, 578 aa), ORF2 (nt 2586–3326, 246 aa), and ORF3 (nt 3326–4051, 242 aa), were identified in the 4.6 kb DNA. BLAST comparison showed that the amino acid sequence of ORF2 shares 54% identity and 71% positives to the NH_3-dependent NAD^+ synthetase of *Mycoplasma genitalium* (GenBank accession number U39720) and 40% identity and 62% positives with a probable NH_3-dependent NAD^+ synthetase of *M. pneumoniae* (*31*). The ORF3 sequence shares 58% identity and 72% positives with the spore coat protein of *B. subtilis* (GenBank accession number Z99118) and 60% identity and 70% positives with the conserved hypothetical protein of *Treponema pallidum* (GenBank accession number AE001224), without any similarity to mycoplasma proteins in the databank. The DNA sequences upstream of ORF1 were also analysed, however, no probable function can be assigned yet.

DISCUSSION

The present study focused on the recombinant clone expressing P60 recognized by rabbit anti-*M. hyopneumoniae* antiserum. The 4.6 kb *Eco*RI fragment was sequenced and analyzed. Using the mycoplasma codons, three open reading frames, ORF1 (from nt 676 to nt 2412, 578aa), ORF2 (nt 2586–3326, 246 aa), and ORF3 (nt 3326–4051, 242 aa), were identified in the 4.6 kb DNA (Figs. 1 and 2). The results of site-directed mutagenesis and Western blot analysis strongly suggest that the TGA_{2231} is used as a tryptophan codon in *M. hyopneumoniae* and the monospecific antibody Ab_{60} indeed can recognize the P72 protein band from *E. coli* DH5α harboring pTLC46M, which is exactly the same size protein as appears among the total proteins of *M. hyopneumoniae*. BLAST comparison revealed that ORF1 encodes P72 and may function as enzyme I of the phosphoenolpyruvate–sugar phosphotransferase system. This biological function was further confirmed by complementation studies. The nearby ORF2 and ORF3 may encode a probable NH_3-dependent NAD^+ synthetase (*31*) and a conserved protein of unknown function, respectively. However, the arrangement of ORF1 (codes for the *ptsI* gene), ORF2 (codes probably for a NH_3-dependent NAD^+ synthetase), and ORF3 (codes for a protein of unknown function) is quite different from the reported dicistronic operon (*ptsI-crr*) in *M. capricolum* (*24*), the linked *ptsH*, *ptsI*, and *crr* genes in *E. coli* (*29*) and the linked *ptsH* and *ptsI* genes in *S. mutans* (*25*). In *E. coli*, the promoter sequences have been compiled and analyzed, and some highly conserved bases have been found to be 5′-TTGACA-3′ around the –35 region and 5′-TATAAT-3′ around the –10 region, with an allowed spacing of 15 to 21 nt between the two conserved regions (*23*). Through sequence comparison, a very similar –35 region (5′-TTGATA-3′), and a much less conserved –10 region (5′-TAAATA-3′), with 13 nt spacing were predicted for the PTSI gene of *M. hyopneumoniae* (Fig. 2). The critical assignments of these regulatory sequences usually requires thorough mutation studies. However, a clear transcription start position (tsp) gives strong support to the prediction. The *in vivo* transcription initiation sites were determined to be in nt 655 (G) and nt 661 (A), in *M. hyopneumoniae* and in *E. coli*, respectively. This is in agreement with the findings that for most *E. coli* promoters, initiation with a purine is highly preferred (*23*). The spacings between the –10 region and the transcription start site are usually varied from 7 to even 41 nucleotides (*15*). In the present study, the spacings between the –10 region and tsp are 14 and 21 nucleotides for *M. hyopneumoniae* and *E. coli*, respectively.

The amino acid sequences of PTSI proteins from *S. mutans*, *B. subtilis*, *L. lactis*, *M. hyopneumoniae*, *S. typhimurium*, *E. coli*, and *M. capricolum* were aligned and compared by the GeneWorks program. It has been suggested that enzyme I is organized into two domains of approximately equal molecular weight (*28*), EI-N and EI-C (the NH_2- and COOH-terminal domains, respectively).

As shown in Fig. 5, the EI-Cs are more conserved than EI-Ns and all the EI-Ns contain the highly conserved active site His-189 (the diamond symbol). This is consistent with the suggestion that the conserved flexible EI-C domain may play a key role in regulating enzyme I and its catalytic properties with respect to phosphoenolpyruvate (PEP), while the compact EI-N domain contains the active site His-189 and interacts with HPr, the histidine containing phosphoryl carrier protein (*28*, *32*). Sequence comparison also revealed that the Trp-502s are highly conserved (the star symbol in Fig. 5). Our complementation data indicate that P72, but not P60 (which has only 501 amino acids), can complement the *ptsI* mutation, further confirming the critical role of Cys-506 (the diamond symbol) in phosphotransfer coupling to HPr (*25*).

The nucleotide sequences and amino acid sequences of enzyme I of *S. mutans*, *B. subtilis*, *L. lactis*, *M. hyopneumoniae*, *S. typhimurium*, *E. coli*, and *M. capricolum* were also analyzed as evolutionary markers by the GeneWorks program. Phylogenetic trees based on UPGMA analysis were constructed using (A) the DNA sequences of the PTSI genes, and (B) the amino acid sequences of PTSIs.

The results of the UPGMA tree analysis of PTSI nucleotide sequences are not in good agreement with the current classification of these microorganisms (*20*). The situation is reconciled as the tree which was generated by amino acid sequences separates the Gram negative from Gram positive bacteria and groups mycoplasmas with the Gram positive bacteria.

The bacterial phosphoenolpyruvate-sugar phosphotransferase system (PTS) is a transport and signal transduction system that is ubiquitous in bacteria but not in eukaryotes. It catalyzes the uptake and phosphorylation of carbohydrates and is involved in signal transduction, chemotaxis (*33*), and allosteric regulation of metabolitic enzymes and transporters. Currently, the unique bacterial phosphotransferase systems provide potential new targets for developing novel antibacterial agents (*34*). Many studies have also shown that catabolic repression plays a role in the expression of virulence factors, and it has been suggested that the expression of virulence genes is controlled by the PTS *via* catabolite repression (*35*). Blocking the PTS pathway may deplete phosphoenolpyruvate, repress the catabolite controlled genes and carbohydrate transport, and should compromise cell growth and infectivity. It seems reasonable that a PTSI mutant could be designed and constructed as a potential recombinant vaccine to prevent swine enzootic pneumoniae. On the other hand, current studies indicate that the SPF pig challenged with *M. hyopneumoniae* pro-

duced antiserum against the intracellular PTSI, but only one out of twelve tested farm animals which had been previously treated with a traditional vaccine showed a response to the antigen. Therefore, further evaluation is needed to develop recombinant protein vaccines based on the PTSI antigen.

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