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# Recombinant 46-Kilodalton Surface Antigen (P46) of *Mycoplasma hyopneumoniae* Expressed in *Escherichia coli* Can Be Used for Early Specific Diagnosis of Mycoplasmal Pneumonia of Swine by Enzyme-Linked Immunosorbent Assay

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**The 46-kDa surface antigen (P46) is the early and species-specific immunogenic protein of *Mycoplasma hyopneumoniae*. Three TGA codons encoding tryptophan in the P46 gene were replaced with TGG by an in vitro mutagenesis technique. The mutated P46 gene was expressed in *Escherichia coli* by using the chelating peptide tag system. The purified recombinant P46 was successfully used in an enzyme-linked immunosorbent assay for detection of antibodies against *M. hyopneumoniae* in swine serum. It did not cross-react with sera from swine infected with *Mycoplasma flocculate*, *Mycoplasma hyorhinis*, or *Mycoplasma hyosynoviae*. With this method, mycoplasmal pneumonia of swine was detectable within 2 weeks after infection.**

*Mycoplasma hyopneumoniae* is a causative agent of mycoplasmal pneumoniae of swine (MPS). It is widespread and causes economic losses in the swine industry through stunted growth caused by poor feeding efficiency and susceptibility of swine to infection by other pathogenic organisms (17). Early diagnosis and isolation of infected pigs from the herd are most effective in preventing the spread of this disease. A reliable diagnosis of *M. hyopneumoniae* infection is necessary for control of MPS. Several serological tests, such as the complement fixation test (14), indirect hemagglutination test (3), and enzyme-linked immunosorbent assay (ELISA) (1, 2, 15), have been developed to demonstrate antibodies to *M. hyopneumoniae* in the sera of diseased pigs, but these techniques were not completely satisfactory because of cross-reaction with other swine mycoplasmas. The double-sandwich ELISA method with a monoclonal antibody that recognized the 46-kDa surface antigen (P46) of *M. hyopneumoniae* was used successfully for the diagnosis of MPS (12). P46 of *M. hyopneumoniae* was considered one of the species-specific surface antigens. Antibodies against P46 were detected most often in the serum of pigs experimentally infected with *M. hyopneumoniae* from the early phase (13). Therefore, it proved to be an excellent marker of MPS. However, preparation of the antigens from *M. hyopneumoniae*, which is an essential part of the double-sandwich ELISA, is time-consuming and costly because it is difficult to cultivate this strain (14, 22).

Recently, we succeeded in isolating the P46 gene from *M. hyopneumoniae* (4). It was revealed that the coding region of the P46 gene was 1,260 bp long, containing three TGA codons, encoding tryptophan. It is known that the TGA codon is translated as a tryptophan rather than as a translational stop in mycoplasmas (6, 16, 23). It has been demonstrated that mycoplasmal genes not containing TGA codons were expressed in *Escherichia coli* (5, 7). Also, several attempts were made to express some genes containing TGA in special host bacteria, such as the *E. coli* suppressor mutant system (20) and the mycoplasmal system (10).

In the present study, we investigated whether the *M. hyopneumoniae* P46 gene in which the TGA codons were replaced with TGG by in vitro mutagenesis could be expressed in *E. coli* by using the maltose-binding protein fusion and chelating peptide tag system for rapid purification of the products. Furthermore, these recombinant proteins were used in the ELISA for specific detection of antibodies against *M. hyopneumoniae*.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** *M. hyopneumoniae* ATCC 25934 (strain J) was grown in BHL medium containing specific-pathogen-free (SPF) horse serum (22). *E. coli* JM109, MV1184, BW313, and BMH71-18 *mutS* were propagated in LB medium (18). Plasmid pURR126 was constructed by insertion of the 5.0-kb *Hin*dIII fragment of *M. hyopneumoniae* chromosomal DNA containing the P46 gene. Expression vector pQE9 (Quiagen Inc., Chatsworth, Calif.) was employed for expression of P46 in *E. coli* (19).

**In vitro mutagenesis.** The accession number for the complete nucleotide sequence of the P46 gene in the DDBJ, EMBL, and GenBank nucleotide sequence databases is D16682 (4). The method of Kunkel et al. was used for site-directed mutagenesis (8) with the Mutan-K kit (Takara Shuzo Co., Ltd., Japan) according to the manufacturer's instructions. Oligonucleotide primers M1 (GGGCACTAATCC ATCGAGGATTA), M2 (GCTGAGTGAGCCAGT TATTTTGTG), and M3 (TTCCATAATTCCATCCTGGGACA) were synthesized and used for mutagenesis (Fig. 1A).

**Construction of expression plasmids.** The coding region of the P46 gene was amplified by PCR. The sequences of the primers used for construction of the chelating peptide-tagged P46 were 5′-GGAAGGGATCCACTTCAGATTCTA AACCACAAGCCGA-3′ (P1, corresponding to downstream from Gly-37 of the P46 open reading frame [ORF]) and 5′-GGAAGCTGCAGTTAACTTTTA GAAATTTTAGGCATC-3′ (P2, corresponding to the C-terminal coding region and 3′-flanking region of the P46 ORF). These primers were designed so that the P46 ORF devoid of signal sequence would be flanked by *Bam*HI (5′) and *Pst*I (3′) sites. The amplified fragment was inserted into the *Bam*HI and *Pst*I sites of plasmid pQE9 so that P46 was in the same translational frame as the N-terminal affinity tag, consisting of the six adjacent histidine residues (6 × His) of the vector (Fig. 1B). The resulting plasmid, pHM46, was used for expression of the recombinant 6 × His-tagged P46 (His-tagP46) in *E. coli*.

**Purification of His-tagP46.** Plasmid pHM46 was introduced into *E. coli* JM109 by transformation. The cells induced by IPTG (isopropylthiogalactopyranoside) were harvested, resuspended in lysis buffer (6 M guanidine hydrochloride, 0.1 M

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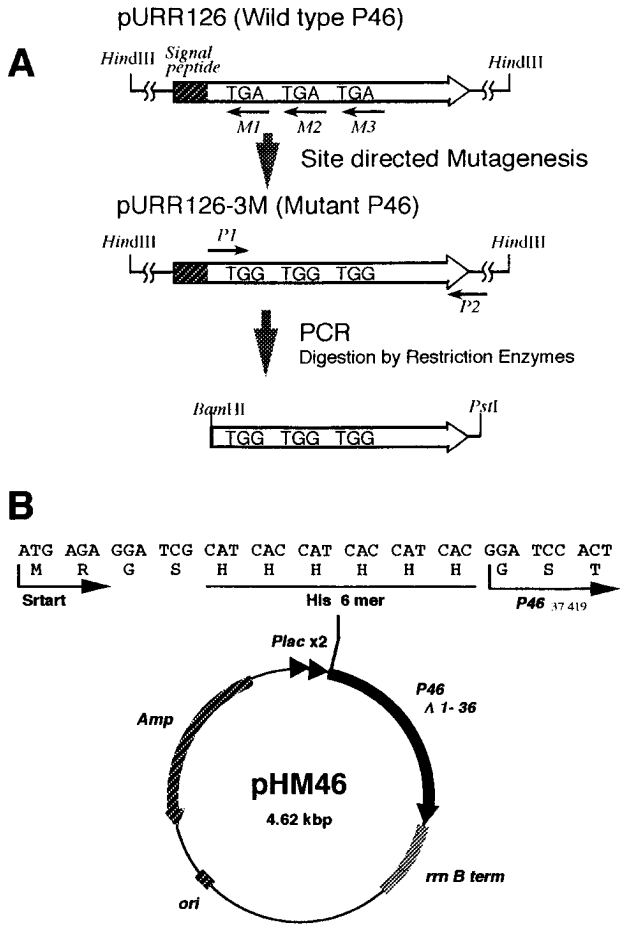


FIG. 1. Construction of pHM46. (A) Three TGA codons in pURR126 were replaced with TGG in pURR126-3M. (B) The coding region of P46 (without a signal peptide) was amplified by PCR and inserted after the 6 × His-tagged extension of pQE9 to construct pHM46 carrying the His-tagP46 gene. Small arrows show the primers used (see Materials and Methods).

$NaH_2PO_4$, 10 mM Tris [pH 8.0]) and shaken by end-over-end rotation for 1 h at room temperature. The extract was clarified by centrifugation at 15,000 rpm for 30 min. The supernatant was loaded onto an $Ni^{2+}$ chelate adsorbent column (Ni-NTA-agarose; Quiagen Inc.) (21). The column was washed with buffer A (8 M urea, 0.1 M $NaH_2PO_4$, 10 mM Tris [pH 6.8]) and eluted with buffer B (the same composition as column buffer but pH 6.0). The fractions containing recombinant His-tagP46 were detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. The pooled fractions were dialyzed against Tris-buffered saline (TBS)–0.5% Tween 20.

**Electrophoresis and immunoblot staining.** SDS-PAGE of proteins was performed with the system of Laemmli (9). Proteins separated on gel were transferred to a nylon membrane by a semidry electrotransfer system (anode buffer: 0.3 M Tris, 20% methanol [pH 10.4]; cathode buffer: 25 mM Tris, 40 mM 6-amino-*n*-hexanoic acid [pH 9.4]; 10 V, 30 min). Anti-P46 monoclonal antibody (MAb) 14-1-1 and alkaline phosphatase-conjugated anti-mouse immunoglobulin A (IgA) and IgG were used for detection of P46. Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were employed as enzyme substrates.

**Antigen, sera, and MAb.** Authentic antigen for the double-sandwich ELISA was prepared by Tween 20 solubilization from *M. hyopneumoniae* as described previously (12). Hyperimmune sera against swine mycoplasmas were prepared by immunizing SPF pigs known to be free of mycoplasmas. Convalescent-phase sera were obtained from hysterectomy-produced colostrum-deprived pigs infected with *M. hyopneumoniae* experimentally. Details of the preparation of these hyperimmune sera and experimental infection have been reported previously (14). An MAb against P46, 14-1-1 (IgA isotype), was used for the double-sandwich ELISA (12).

**ELISA.** The double-sandwich ELISA was performed as follows (12). Wells of polystyrene microplates were coated with diluted MAb 14-1-1 (ascites fluid). After being washed with PBST (phosphate-buffered saline, 0.05% Tween 20), the antigen extracted from *M. hyopneumoniae* or recombinant protein (at an appropriate dilution) was added and incubated for 2 h at room temperature. The wells were washed and incubated with swine serum (1:100 or 1:1,000 dilution) for 2 h at room temperature. Peroxidase-conjugated rabbit anti-swine IgG (light and heavy chains; 1:1,000 dilution) was used for detection of antibody, and 2,2′-azinobis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) was used as a peroxidase substrate. The dilutions of serum and conjugant were made in PBST containing 5% fetal calf serum.

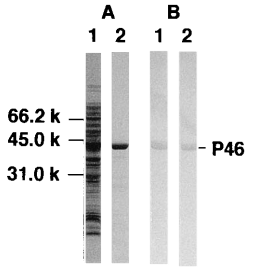


FIG. 2. Purification of His-tagP46. Samples were separated by SDS–5 to 20% PAGE and stained with Coomassie blue R-250 (A) and immunostained with anti-P46 MAb 14-1-1 (B). Lane 1, cell lysate of induced *E. coli* containing pHM46; lane 2, proteins separated by $Ni^{2+}$ column chromatography. The positions of P46 and size markers (in kilodaltons) are indicated.

## RESULTS

**Conversion of TGA codons to TGG.** In mycoplasma species, the TGA codon is utilized for the amino acid tryptophan rather than as a translational stop (6, 23). The P46 gene of *M. hyopneumoniae* has three TGA codons encoding tryptophan. For production of the full-length P46 protein in *E. coli*, it is necessary to replace TGA with TGG, which is universally recognized as a tryptophan codon. We adopted in vitro mutagenesis techniques to replace the bases (Fig. 1A). Correct base changes were confirmed by DNA sequencing analysis.

**Expression of the His-tagP46 in *E. coli*.** A 1.1-kb DNA fragment containing the modified P46 gene devoid of its putative promoter and signal sequence (P46Δ1-36) was amplified by PCR and subcloned into the *Bam*HI and *Pst*I sites of the expression vector pQE9. The resulting plasmid, pHM46 (Fig. 1B), was used for expression of the recombinant P46Δ1-36 protein containing an N-terminal affinity tag consisting of six His residues, which allows purification by $Ni^{2+}$ affinity column chromatography. The tandem *lac* promoters were used to drive transcription of the gene. The recombinant 6 × His-tagged P46Δ1-36 protein (His-tagP46) was induced by addition of IPTG to the culture medium. Four hours after induction with IPTG, a significant amount of the recombinant protein accumulated in the cells. An inclusion body was not observed. The recombinant protein was strongly bound to the anti-P46 MAb 14-1-1. The whole-cell proteins were solubilized with 6 M guanidine hydrochloride and applied to an $Ni^{2+}$ column for purification of the recombinant protein. The recombinant His-tagP46 obtained was >90% pure in SDS-PAGE (Fig. 2).

**ELISA with recombinant protein.** The immunological reactivity of the purified recombinant protein was compared with that of authentic P46 of *M. hyopneumoniae* by the double-sandwich ELISA method. The Tween 20-solubilized antigen of *M. hyopneumoniae* was conjugated with immobilized MAb 14-1-1 in the wells and washed to purify the authentic P46. The purified recombinant P46 reacted with six convalescent-phase sera from swine infected with *M. hyopneumoniae* but did not react with sera from pigs infected with other swine mycoplasmas or from SPF pigs (Fig. 3).

The early antibody response against *M. hyopneumoniae* was monitored by the double-sandwich ELISA with recombinant

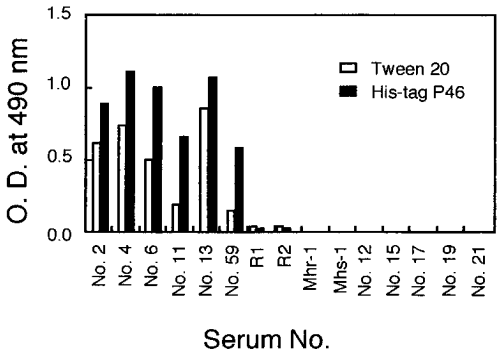


FIG. 3. Comparison of immunoreactivities between *M. hyopneumoniae* Tween 20-solubilized authentic antigen (open bars) and recombinant His-tagP46 (solid bars) fusion by double-sandwich ELISA for detection of antibodies against *M. hyopneumoniae*. Convalescent-phase sera were obtained from swine infected with *M. hyopneumoniae* (samples 2, 4, 6, 11, 13, and 59), *M. flocculare* (R1 and R2), *M. hyorhinis* (Mhr-1), and *M. hyosynoviae* (Mhs-1). Serum samples 12, 15, 17, 19, and 21 were from SPF herds. Serum samples were used at a 1:100 dilution for the assay. O.D., optical density.

P46 and the Tween 20-solubilized authentic antigen of *M. hyopneumoniae* (Fig. 4). These data demonstrated that the double-sandwich ELISA with recombinant P46 could detect antibodies against *M. hyopneumoniae* as well as with the Tween 20-solubilized authentic antigen.

## DISCUSSION

ELISA is considered the most useful test for detection of antibodies to *M. hyopneumoniae*. Many attempts have been made to purify the specific antigen from *M. hyopneumoniae* (1, 15). However, it is very difficult to remove cross-reactivities against other swine mycoplasmas, especially against *Mycoplasma flocculare*. The ELISA double-sandwich method with an MAb that recognizes P46 was used successfully for specific detection of antibodies against *M. hyopneumoniae* (12). P46 is an early and strongly immunogenic 46-kDa membrane-associated protein of *M. hyopneumoniae* (13).

Recently, we succeeded in isolating the P46 gene, which had a 1,260-bp-long coding region containing three TGA codons for tryptophan (4). It has also been reported that a TGA codon encodes tryptophan in other several mycoplasmas (6, 23). The presence of these TGA codons in the gene would prevent production of the complete protein in *E. coli*. Therefore, we used an in vitro mutagenesis technique to replace the TGAs with the universal TGG tryptophan codon.

The base-replaced gene was introduced into the 6 × His-tagged fusion system by using the pQE9 plasmid vector for expression of P46 to improve the purification step. A short peptide consisting of six His residues, which did not show immunogenicity, was introduced into the N-terminal part of the P46 ORF. The recombinant His-tagP46 was purified to 90% purity by a single $Ni^{2+}$ affinity chromatography step. This construct was extremely useful for production of the recombinant P46 antigen.

The purified recombinant antigen can be used in the ELISA for specific detection of antibodies against P46 of *M. hyopneumoniae*. Antisera from pigs which were infected with other swine mycoplasmas do not react against these recombinant antigens (Fig. 3). The early antibody response of the His-tagP46 was similar to that of authentic P46 (Fig. 4). These data showed that the ELISA for P46 antibodies gives excellent specificity for the diagnosis.

The indirect ELISA, without the MAb against P46, was also applicable with this purified antigen (Fig. 4). The background level of the indirect ELISA was about 10 times higher than that of the double-sandwich method. Further purification of the antigen may reduce the background in this simpler method.

The His-tagged recombinant P46 showed almost the same reactivities as the native P46 from *M. hyopneumoniae* in the ELISA detection of P46 antibodies even though it does not contain the N-terminal portions (residues 1 to 37). The N-terminal lipoprotein portion of P46 (4, 11) may associate with the membrane and hide from the swine immune system, not being recognized as an epitope.

In this study, we have successfully demonstrated production of *M. hyopneumoniae* P46 in *E. coli* and specific detection of antibodies against *M. hyopneumoniae* by ELISA with a recombinant P46. It improved the reproducibility and simplicity of preparation of an ELISA antigen for diagnosis of *M. hyopneumoniae* infection. With this early and specific method, infection with *M. hyopneumoniae* is detectable within 2 weeks after infection (Fig. 4). This remarkable species-specific detection can prevent a wrong diagnosis. We consider that it contributes to maintaining an SPF herd of swine and will increase the productivity of a pig farm.

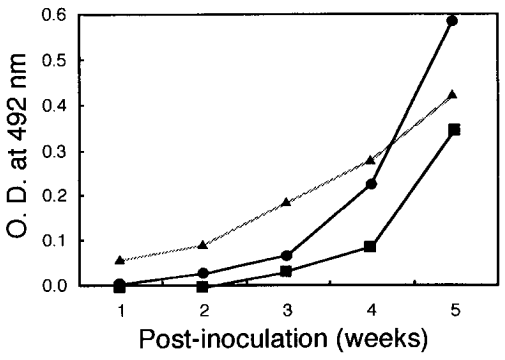


FIG. 4. Monitoring of early antibody response against *M. hyopneumoniae* by the double-sandwich ELISA with recombinant His-tagP46 (●) and Tween 20-solubilized authentic antigen (■). Serum samples were used at a 1:100 dilution for the assay. These data indicate the mean optical density (O.D.) of sera from seven pigs inoculated with *M. hyopneumoniae*. In the indirect ELISA (▲), a His-tagP46-coated plate was used.

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