

## Cloning and Expression of a Species-Specific Early Immunogenic 36-Kilodalton Protein of *Mycoplasma hyopneumoniae* in *Escherichia coli*

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*Mycoplasma hyopneumoniae*, the etiologic agent of porcine enzootic pneumonia, synthesizes a 36-kDa protein which is an early and strong immunogenic factor in experimentally and naturally infected swine. The gene encoding this protein was cloned by screening a gene library of *M. hyopneumoniae* DNA with rabbit hyperimmune serum made against whole *M. hyopneumoniae* cells and convalescent-phase swine serum. Analysis of the recombinant protein expressed in *Escherichia coli* by immunoblot techniques showed that the protein is expressed in *E. coli* in its full length and does not cross-react with proteins from *M. flocculare* or *M. hyorhinitis*. Genetic analysis showed that the gene was expressed from the *lac* promoter of the vector and seems to be translationally initiated from its own ribosome binding site. Subcloning in a transcriptional fusion vector to optimize expression resulted in production of the 36-kDa protein in *E. coli* at levels up to 30% of total protein.

*Mycoplasma hyopneumoniae* is the etiologic agent of enzootic pneumonia of swine (20, 23). In spite of the fact that this is one of the most important diseases affecting pigs in the world, knowledge of the pathogenicity (18) and the virulence factors involved is very poor. A protein from *M. hyopneumoniae* which has an approximate molecular weight of 54,000 has been reported to have a cytopathic effect on cultured MRC-5 human lung fibroblasts (7). Direct evidence for specific proteins involved in virulence of *M. hyopneumoniae* is still lacking. The absence of gene transfer systems for the study of *M. hyopneumoniae* and the difficulty in obtaining single colonies, as well as tedious growth procedures which require highly serum-enriched media, render the genetic and biochemical analyses of *M. hyopneumoniae* and the study of the factors involved in pathogenicity of enzootic pneumonia very difficult. In addition, the serological differentiation of *M. hyopneumoniae* (12) from other nonpathogenic porcine mycoplasmas is hindered by cross-reactions (4, 25), mainly with the related, but apparently apathogenic, common porcine mycoplasma *M. flocculare*.

A better understanding of the nature of predominant antigens of *M. hyopneumoniae* will not only be useful in the attempt to detect factors involved in virulence and the study of pathogenicity, but also for serological differentiation of porcine *Mycoplasma* species and for the development of vaccines. To study the pathogenicity of *M. hyopneumoniae*, we are attempting to clone genes of *M. hyopneumoniae* that encode proteins responsible for a strong immunogenic response in infected animals. In this report, we describe the cloning of an *M. hyopneumoniae* gene encoding a 36-kDa protein and the expression of this protein in *Escherichia coli*. Using sera from experimentally infected pigs taken at various times after infection, we showed that the protein elicits an early and strong immunogenic reaction.

### MATERIALS AND METHODS

**Organisms and growth conditions.** The mycoplasma strains used were *M. hyopneumoniae* NTCC 10110, *M. hyopneumoniae* BQ 14 (French field strain), *M. hyorhinitis* BTS 7 (ATCC 10130), and *M. flocculare* MS42 (NCTC 10143).

*M. hyopneumoniae* and *M. flocculare* were both grown in Friis medium (6), and *M. hyorhinitis* was grown in Berne medium (2) at 37°C and harvested at a pH of 6.9 ( $5 \times 10^7$  cells per ml) by centrifugation at  $20,000 \times g$  for 20 min at 4°C. The cells were washed subsequently three times in 0.25 M NaCl and resuspended in the same solution in 1/100 of the original volume. *E. coli* XL1-blue *endA* *hsdR17* *supE44*  $\lambda^-$  *recA1*  $\Delta$ (*pro-lac*) [F' *proAB lacI<sup>q</sup>ΔM15 Tn10*(Tet<sup>r</sup>)] (3) was grown in highly concentrated LB broth (5 g of NaCl, 20 g of yeast extract [Difco], 35 g of tryptone, and 1 ml of 5 M NaOH per liter) supplemented with tetracycline (25 μg/ml), 0.2% maltose, and 10 mM MgSO<sub>4</sub> to an A<sub>650</sub> of 0.6 at 37°C. The cells were centrifuged at  $1,000 \times g$  for 5 min and gently resuspended in 0.5 volume of 10 mM MgSO<sub>4</sub> as described by Maniatis et al. (10). Induction of the *lacZ* promoter was obtained by adding 0.1 mM (final concentration) isopropyl-β-D-thiogalactosidase (IPTG; Biofinex, Praroman, Switzerland) to cultures at an A<sub>650</sub> of 0.2 and subsequent growth to an A<sub>650</sub> of 0.6.

The *M. hyopneumoniae* membrane fraction was prepared as described by Razin and Rottem (16).

**Experimental infection of piglets.** Three specific-pathogen-free hysterectomized piglets, originating from one sow, were inoculated on 2 successive days intratracheally with a 5-ml culture suspension of *M. hyopneumoniae* BQ14 ( $10^8$  CFU/ml). Blood was collected weekly to prepare sera.

**Preparation of antisera.** Rabbit anti-*M. hyopneumoniae* hyperimmune serum was prepared as described earlier (2). Antiserum against recombinant mycoplasma proteins produced in *E. coli* was made in mice. A total of  $2.5 \times 10^9$  sonicated cells of the respective recombinant *E. coli* clone, with complete Freund adjuvant, was subcutaneously in-

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jected in each animal. Two booster injections were made with incomplete Freund adjuvant at 3-week intervals. After 3 months, animals were bled and antiserum was used for immunoblot analysis. Antisera used on immunoblots with extracts of recombinant *E. coli* strains were absorbed with a sonicated extract of *E. coli* XL1-blue cells prior to use, as described by Trevino et al. (22).

**Mycoplasma DNA extraction and construction of the genomic library.** DNA from *M. hyopneumoniae* was prepared as follows. One milliliter ( $5 \times 10^9$  CFU/ml) of *M. hyopneumoniae* suspension was mixed with 100  $\mu$ l of proteinase K (2 mg/ml) (Boehringer, Mannheim, Germany) and 25  $\mu$ l of 25% sodium dodecyl sulfate (SDS); the mixture was incubated for 1 hour at 37°C and extracted twice with an equal volume of phenol saturated with 0.1 M Tris (pH 8.0) and once with chloroform-isoamyl alcohol (24:1). The DNA was then precipitated from the aqueous phase as described by Maniatis et al. (10) and resuspended in 50  $\mu$ l of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). RNase (Boehringer) was added at a final concentration of 50  $\mu$ g/ml.

A 40- $\mu$ l sample of *M. hyopneumoniae* DNA (0.4  $\mu$ g/ $\mu$ l) was sonicated for 1 s with a Branson Sonifier (Branson, Danbury, Conn.) at 6.5 output control. Resulting fragments of 100 to 10,000 bp were made blunt ended by treatment with Klenow-polymerase (Biofinex) and ligated to synthetic 12-mer *Eco*RI linkers (*Eco*RI linkers for  $\lambda$ gt cloning [Boehringer]). The fragments were then heated to 80°C, annealed, cloned in an *Eco*RI-cleaved lambda ZAP II cloning vector (19), and subsequently packaged with commercially available Lambda packaging extracts (Promega, Madison, Wis.). *E. coli* XL1-blue was infected with recombinant lambda ZAP II phages and prepared for screening with rabbit anti-*M. hyopneumoniae* hyperimmune sera, as described by Ausubel et al. (1). Subcloning was made in expression vector pBlue-scriptII SK<sup>-</sup> (Stratagene, La Jolla, Calif.).

**Analysis and screening of proteins in recombinant strain and immunoblotting.** For preparation of samples, 20  $\mu$ l of an *E. coli* broth culture ( $A_{650}$  of 1.0), 20  $\mu$ l of suspended *M. hyopneumoniae*, *M. flocculare*, or *M. hyorhinis* cells ( $5 \times 10^9$  CFU/ml), or 20  $\mu$ l of *M. hyopneumoniae* membrane corresponding to  $5 \times 10^9$  CFU was separated by SDS-polyacrylamide (12.5%) gel electrophoresis as described by Laemmli (9). The Western blot (immunoblot) procedure was carried out by the method of Towbin et al. (21), using 0.2- $\mu$ m nitrocellulose membranes (Bio-Rad Laboratories, Richmond, Calif.). The immunoblot procedure was a slight modification of the method described by Young and Ross (25) and used goat anti-rabbit, anti-swine, or anti-mouse immunoglobulin G (heavy and light chains) phosphatase-labeled conjugate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.). Visualization was performed as described by Knecht and Diamond (8).

**Preparation and in vitro manipulation of recombinant plasmid DNA.** Isolation of plasmid DNA, transformation, restric-

tion endonuclease digestion, ligation, agarose gel electrophoresis, and other standard recombinant DNA techniques were performed as described by Maniatis et al. (10). Insertion mutagenesis by the interposon Omega (14) was carried out as described by Frey and Krisch (5).

**Immunocytochemical analysis of anti-P36 by electron microscopy.** Pellets of *M. hyopneumoniae* were fixed in 2% glutaraldehyde-1.5% paraformaldehyde in phosphate-buffered saline, pH 7.4. Specimens were embedded in Spurr low-viscosity medium. Thin sections (60 nm thick) were cut and processed for electron microscopic immunocytochemistry by the following modification of Jansen's protocol (Jansen Life Science Products, Beerse, Belgium): no etching, incubation with mouse anti-P36 serum (dilution, 1:400) for 1 h, washing and incubation in goat anti-mouse immunoglobulin G conjugated to 15-nm colloidal gold particles (dilution, 1:4), and washing. Serum was diluted in phosphate-buffered saline-Tween 20 (0.5%). All sections were examined and photographed with a Zeiss EM-902 PC at 50 kV. Negative controls were made by omitting mouse anti-P36 serum.

## RESULTS

**Cloning of the gene encoding the 36-kDa *M. hyopneumoniae* protein.** A gene library of *M. hyopneumoniae* was made by cloning randomly generated DNA fragments into the bacteriophage lambda ZAP II expression vector (titer of packaged phages,  $4.5 \times 10^4$  PFU/ $\mu$ l). On plates containing X-Gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galacto-pyranoside), 60% of phage plaques were uncolored, indicating that this proportion contained inserts. Initial immunoscreening of the library with rabbit anti-*M. hyopneumoniae* serum resulted in 5% immunologically reacting plaques. Strongly reacting plaques were isolated and used to infect fresh *E. coli* lawns. The plaques were again screened with rabbit anti-*M. hyopneumoniae* serum and swine anti-*M. hyopneumoniae* serum. Clones reacting with both rabbit and swine sera (36 clones) were converted into the vector pBluescript. Immunoblot analysis of the total proteins of those recombinant *E. coli* strains was made with swine anti-*M. hyopneumoniae* serum. One clone, containing plasmid p36001, showed a distinct and strongly reacting protein band with a molecular weight of 36,000. The expression of this protein was inducible with IPTG. Restriction enzyme analysis showed that the cloned DNA insert in hybrid plasmid p36001 was a 4.2-kb fragment (Fig. 1).

To determine whether the 36-kDa protein, which is expressed from the hybrid plasmid p36001, is a full-length *M. hyopneumoniae* protein, antibodies against total proteins of an IPTG-induced *E. coli* strain containing plasmid p36001 were made in mice and subsequently absorbed with the host strain containing the vector alone. These antibodies reacted on immunoblots containing SDS-solubilized total proteins of *M. hyopneumoniae* with a 36-kDa protein (Fig. 2, lane a)

FIG. 1. Restriction enzyme map of recombinant plasmid pBluescript containing the *M. hyopneumoniae* insert encoding P36 and subclones. Bold lines represent *M. hyopneumoniae* DNA obtained from *Eco*RI-digested chromosomal DNA cloned into the polylinker *Eco*RI site of lambda ZAP II. The polylinker of pBluescript is shown (▣) (not drawn to scale). Only relevant restriction sites are given. The *lac* promoter of pBluescript is shown (▶). The Omega fragment, with the transcription termination (⊖) and the translation termination (∇), is shown. A part of the plasmid pBluescript is indicated (▣). The following plasmids are shown: p36001 primary isolated clone expressing P36 containing a 4.2-kb *M. hyopneumoniae* fragment; p36002 derived from p36001 by deleting the 2.5-kb *Hind*III fragment and elimination of the *Hind*III site; p36005 derived from p36002 by insertion of the Omega fragment in the *Bam*HI site; p36004 produced by deleting the internal *Hind*III fragment of Omega; p36005 derived from p36002 by deleting a 400-bp *Stu*I-*Hinc*II fragment (produces a truncated P36 protein); pJFF631 made from p36002 by deleting the 300-bp *Sma*I-*Hpa*I fragment, thus placing the vector's *lac* promoter near the beginning of the P36 gene. The location and direction of transcription of the gene encoding P36 are indicated by the open arrow.



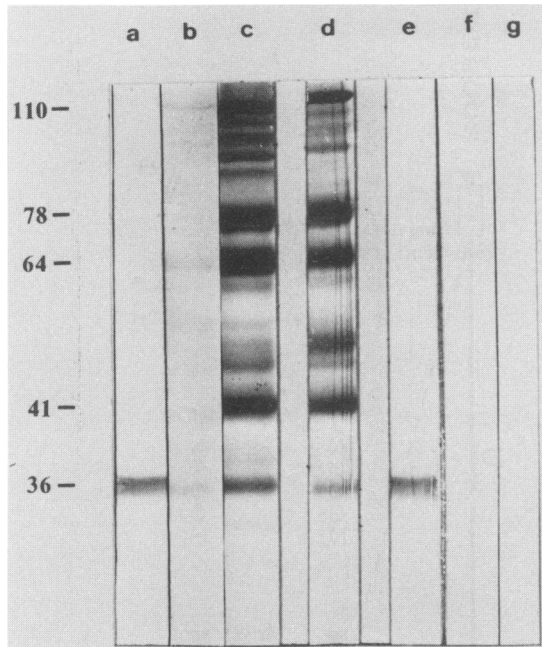


FIG. 2. Immunological specificity of protein P36. The immunoblots (lanes a to e) contain aliquots of total SDS-solubilized *M. hyopneumoniae* proteins and were incubated with the following sera: lane a, mouse anti-P36K serum; lane b, swine anti-*M. hyopneumoniae* convalescent serum from an experimental infection 4 weeks postinfection; lane c, swine anti-*M. hyopneumoniae* convalescent serum from an experimental infection 12 weeks postinfection; lane d, rabbit anti-*M. hyopneumoniae* hyperimmune serum. The immunoblots in lanes e to g contain the following total cell antigens: lane e, *M. hyopneumoniae*; lane f, *M. flocculare*; lane g, *M. hyorhinis* (all incubated against mouse anti-P36 serum). The positions and molecular weights of the major immunogenic proteins of *M. hyopneumoniae* are indicated on the left.

which was also detected when sera from *M. hyopneumoniae*-infected swine were used (lanes b and c), indicating that the cloned 36-kDa protein (designated P36) encoded by plasmid p36001 is expressed at full or near full length and corresponds to the immunogenic 36-kDa protein of *M. hyopneumoniae*.

The gene encoding P36 on plasmid p36001 was subcloned by deleting the 2.5-kb *Hind*III fragment and subsequently removing the *Hind*III site, resulting in plasmid p36002, which has an insert of 1.7 kb. Removal of the *Hind*III site was made by filling in the 5' protruding single-stranded ends with deoxynucleoside triphosphate and Klenow enzyme (10) prior to the ligation. This procedure created an *Nhe*I site at the place of the *Hind*III site. This plasmid can still produce an IPTG-inducible P36 protein (Fig. 3A). To verify whether the gene was transcribed uniquely by the *lac* promoter of the vector and to exclude the possibility that P36 is produced as a fusion protein with part of the expression vector, we cloned the Omega interposon into the *Bam*HI site of p36002 just in front of the cloned fragment (plasmid p36003; Fig. 1). By this procedure, we introduced strong transcription signals and translation stop codons in all three reading frames so that the expression of protein P36 was completely suppressed (Fig. 3B). Removal of the transcription stop signals and the *Sm*<sup>r</sup> *Spc*<sup>r</sup> genes by deleting the internal *Hind*III fragment of the Omega interposon, but leaving the translation stop codons (plasmid p36004; Fig. 1), reestablished the

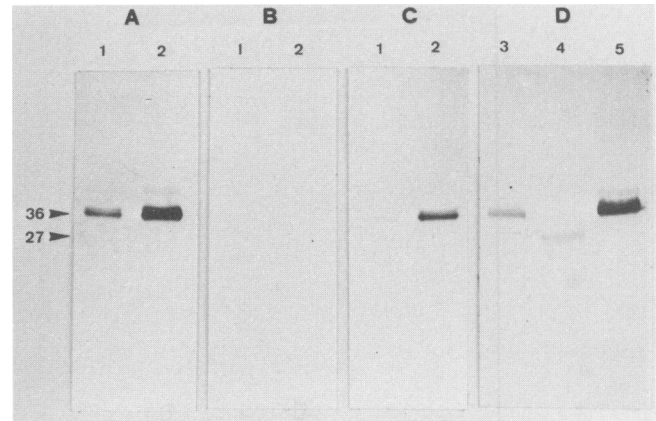


FIG. 3. Analysis of the expression of the cloned gene encoding P36 by immunoblot. (A) Clone p36001; lane 1, not induced with IPTG; lane 2, IPTG induced. (B) Clone p36003 containing the Omega fragment with the translation and transcription termination signals; lane 1, not induced with IPTG; lane 2, IPTG induced. (C) Clone p36004 (only containing the translation stop from Omega); lane 1, not induced with IPTG; lane 2, IPTG induced. (D) Immunoblot analysis of IPTG-induced subclones from p36001; lane 3, 36-kDa protein band of clone p36002; lane 4, 27-kDa truncated protein of clone p36005; lane 5, strong 36-kDa protein P36 band of clone pJFF631.

IPTG-inducible expression of P36 as shown on the immunoblot in Fig. 3C.

Further reduction of the size of the cloned fragment in p36002 by deleting the right part from the internal *Stu*I site to the vector's *Hinc*II site resulted in plasmid p36005 (Fig. 1), which expressed a truncated 27-kDa protein reacting with antibodies against P36 (Fig. 3). This allowed us to localize the end of the P36 gene approximately 250 bp downstream from *Stu*I and its start close to the *Hpa*I site (Fig. 1). Deletion of a 300-bp fragment extending from the *Sma*I site in the vector's polylinker to the *Hpa*I site resulted in plasmid pJFF631. This plasmid produces a strongly IPTG-inducible P36 protein, giving rise to a very strong production of protein P36, contributing up to 30% of total cellular protein (Fig. 3D).

**Immunogenic relevance of P36.** The role played by P36 in the immune response of experimentally *M. hyopneumoniae*-infected piglets was tested by the following experiment. Sera taken from pigs 4 and 12 weeks after experimental infection with *M. hyopneumoniae* were analyzed on immunoblots containing either total proteins from SDS-solubilized *M. hyopneumoniae* cells or the cloned 36-kDa protein expressed in *E. coli*. The immunoblot analysis with the serum taken 4 weeks after infection, using total SDS-solubilized *M. hyopneumoniae* proteins as antigens, shows that the 36-kDa protein which corresponds to P36 is one of the predominant early immunogenic proteins (Fig. 2, lane b). The immunoblot with the serum taken 12 weeks after infection shows that the strong immunogenic reaction of P36 persists (Fig. 2, lane c). The same sera also strongly detected the recombinant P36 protein expressed in *E. coli* (data not shown).

To test the antigenic relatedness of the cloned P36 protein with other porcine mycoplasmas, immunoblots with equal amounts of total SDS-solubilized proteins from *M. hyopneumoniae* and the related species *M. flocculare* and *M. hyorhinis* were made with mouse anti-P36 serum. These experiments showed a strong reaction of anti-P36 serum with the

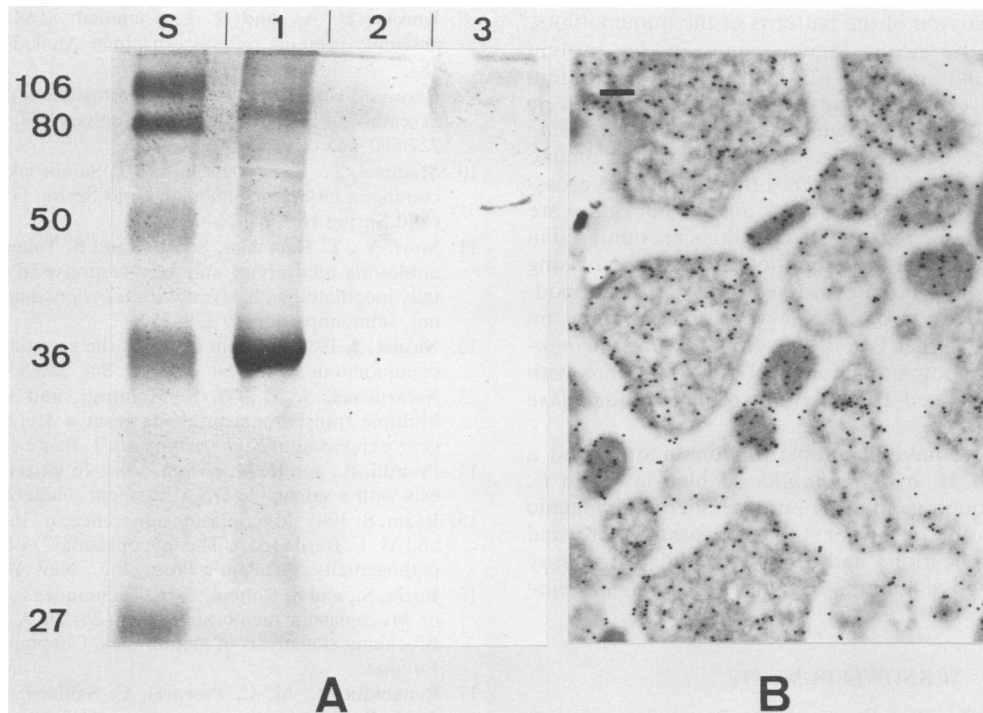


FIG. 4. Localization of P36 in *M. hyopneumoniae* cells. (A) Immunoblot using anti-P36 polyclonal antibodies: lane S, size standard, molecular weights ( $10^3$ ); lane 1, total *M. hyopneumoniae* cells; lane 2, membrane fraction of *M. hyopneumoniae* (amount of proteins used corresponds to 10-fold the amount used in lane 1); lane 3, supernatant of an *M. hyopneumoniae* culture concentrated 50-fold. (B) Immune electron microscopy of *M. hyopneumoniae* cells incubated with the same anti-P36 antibodies. Gold particle size, 15 nm; bar, 0.25  $\mu\text{m}$ .

36-kDa protein of *M. hyopneumoniae* (Fig. 2, lane e) but no reaction at all with proteins of *M. flocculare* or *M. hyorhinis* (Fig. 2, lanes f and g), demonstrating that the P36 antigenic protein is specific to *M. hyopneumoniae*.

**Localization of P36 in *M. hyopneumoniae*.** To localize P36 in or on the *M. hyopneumoniae* cell, we have analyzed a concentrated supernatant of an *M. hyopneumoniae* culture and a membrane fraction and total culture of *M. hyopneumoniae* by SDS-gel electrophoresis and subsequent immunoblotting with mouse anti-P36 serum. While total *M. hyopneumoniae* cells showed a strong reaction with P36, no reaction at all was found in 50-fold-concentrated supernatant or in the membrane fraction of *M. hyopneumoniae* which corresponded to 10-fold the amount of proteins used for total culture (Fig. 4A). P36 therefore seems to be located intracellularly in *M. hyopneumoniae* cells grown in culture. This result was confirmed by immunocytochemical analysis of thin sections of *M. hyopneumoniae* cells, using mouse anti-P36 serum and anti-mouse immunoglobulin G-gold conjugate. Inspection of the electron microscope pictures showed an equal distribution of the gold particles on the *M. hyopneumoniae* cells but no particular concentration on the membranes and no gold label in between the cells (Fig. 4B).

#### DISCUSSION

We have cloned and expressed in *E. coli* the gene for a predominant immunogenic 36-kDa protein (designated P36) from *M. hyopneumoniae*. Immunoblot analysis with total native *M. hyopneumoniae* proteins as antigens and antibodies made against recombinant P36 showed that the cloned P36 gene expressed in *E. coli* has the same length as in *M. hyopneumoniae*. We conclude, therefore, that this gene does

not contain internal in-frame UGA codons which have been found in other mollicutes to encode tryptophan (17, 24), unless such a codon would be next to the P36 C terminal. The transcription of the cloned P36 gene in *E. coli* was totally inhibited when strong transcription stop signals from the Omega interposon were cloned between the *lac* promoter of the vector and the cloned DNA fragment (plasmid p36003). The cloned DNA therefore contains no promoter for P36 that is recognized in *E. coli* in the DNA approximately 500-bp upstream from the beginning of the P36 gene. P36 might be transcribed in its original host from a promoter of a multigene operon located upstream (outside) of the cloned DNA fragment or from a promoter that is not recognized in *E. coli*. The introduction of translation stop signals in all three reading frames at the junction vector/insert, however, had no effect on P36 expression. The gene encoding P36 therefore must contain translation initiation signals that are recognized efficiently in *E. coli*. The correct positioning of the beginning of the P36 gene near the *lac* promoter of the pBluescript vector allowed us to obtain very high amounts of P36 in *E. coli*.

Notarnicola et al. (13) have recently found multiple translational initiation on a cloned gene from *M. hyorhinis* which was expressed in *E. coli*, indicating that translational initiation sites used in *E. coli* are not active in mycoplasmas and suggesting differences between translational regulatory signals of mycoplasma and eubacteria. In contrast, our results on the expression of P36 of *M. hyopneumoniae* in *E. coli* have not shown such multiple initiations. From subcloning experiments we know that the signal for initiating P36 translation in *E. coli* must lie near the beginning of the gene and could be congruent or even identical to that used in *M. hyopneumoniae*.

From the comparison of the patterns of the immunoblots, we assume that the strong immunogenic 36-kDa protein described by Young and Ross (25) and the 38-kDa protein reported by Mori et al. (11) correspond to P36. However, Young and Ross (25) have shown that an *M. hyopneumoniae* 36-kDa protein serologically cross-reacts with hyperimmune serum raised against *M. flocculare*. Immunological cross-reactions between *M. hyopneumoniae* and *M. flocculare* are well-known and are the reason for difficulties encountered in serological differentiation between these two species. Using the recombinant P36 protein expressed in *E. coli* or antibodies raised against this protein, we were able to show on immunoblot analysis that P36 is specific to *M. hyopneumoniae* and does not show immunological cross-reactions with proteins from the related *Mycoplasma* species *M. flocculare* and *M. hyorhinis*.

In conclusion, we have been able to clone and express a 36-kDa protein of *M. hyopneumoniae* at high levels in *E. coli*. That this protein is an early and specific immunogenic factor makes it useful for further studies of pathogenicity and for diagnostic applications and a good candidate to assay induction of protective immunity against *M. hyopneumoniae* infections.

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