Monoclonal Antibodies to *Escherichia coli*-Expressed P46 and P65 Membranous Proteins for Specific Immunodetection of *Mycoplasma hyopneumoniae* in Lungs of Infected Pigs

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Received 18 July 2002/Returned for modification 18 November 2002/Accepted 22 January 2003

The P46 and P65 proteins of *Mycoplasma hyopneumoniae* are two membranous proteins carrying species-specific antigenic determinants. Based on the genomic sequence of the reference strain ATCC 25934, primers were designed for PCR amplification of the genes encoding entire P46 (1,260 bp) and P65 (1,803 bp) and N-terminally truncated P65\(_{ac}\) (1,200 bp). These primers were shown to be specific to *M. hyopneumoniae* since no DNA amplicons could be obtained with other mycoplasma species that commonly colonize the porcine respiratory tract. Both amplified genes were then cloned into the pGEX-4T-1 vector to be expressed in *Escherichia coli* cells as recombinant fusion proteins with glutathione S-transferase (GST). Prior to generation of expression constructs, TGA nonsense codons, exceptionally used for tryptophan residues by *M. hyopneumoniae*, had been converted to TGG codons by PCR-directed mutagenesis. Following induction by IPTG (isopropyl-β-D-thiogalactopyranoside), both GST-P46 and GST-P65\(_{ac}\) recombinant fusion proteins were recovered by disrupting transformed cells by sonication, purified by affinity chromatography, and then cut with thrombin to release the P46 and P65\(_{ac}\), moieties. The enriched *E. coli*-expressed P46 and P65\(_{ac}\) were used to immunize female BALB/c mice for the generation of anti-P46 and anti-P65\(_{ac}\), monoclonal antibodies (MAbs). The polypeptide specificities of MAbs obtained was confirmed by Western blotting with cell lysates prepared from the homologous strain. Cross-reactivity study of the anti-P46 and anti-P65\(_{ac}\) MAbs towards two other *M. hyopneumoniae* reference strains (ATCC 25095 and J strains) and Quebec field strains that had been isolated in culture, suggested that the MAbs obtained against both membranous proteins were directed against highly conserved species-specific epitopes. No reactivity to other mycoplasma species tested was demonstrated. Clinical signs suggested that the MAbs obtained against both membranous proteins were directed against highly conserved species-specific epitopes. No reactivity to other mycoplasma species tested was demonstrated. Clinical signs and lesions suggestive of enzootic pneumonia were reproduced in specific-pathogen-free pigs that had been inoculated intratracheally with a virulent Quebec field strain (IAF-DM9827) of *M. hyopneumoniae*. Both anti-P46 and anti-P65\(_{ac}\), MAbs permitted effective detection by indirect immunofluorescence and indirect immunoperoxidase assay of *M. hyopneumoniae* in, respectively, frozen and formalin-fixed, paraffin-embedded lung sections from pigs that were killed after the 6- to 7-week observation period.

*Mycoplasma hyopneumoniae* is the causative agent of enzootic pig pneumonia, a disease found on pig farms worldwide and characterized by high morbidity and low mortality rates (15, 22). Coughing is the main clinical sign, but retarded growth and poor food conversion may result in considerable economic losses (22, 26). This microorganism predisposes pigs to secondary infections that increase the mortality rates, such as infections by porcine reproductive and respiratory syndrome virus and swine influenza virus (33). The diagnosis of *M. hyopneumoniae* is usually done by PCR, cultivation of the organism in enriched Friis medium, or immunofluorescence tests performed on frozen thin lung sections (1, 3, 5, 6, 17, 19, 25). The culture of this fastidious bacteria and its identification may take up to 1 month. Contamination with *Mycoplasma hyorhinis* and *Mycoplasma flocculare*, both considered to be nonpathogenic species, is frequently observed. These less fastidious nonpathogenic species may often overgrow *M. hyopneumoniae* in primary isolation attempts (20), from which arises the necessity to discriminate among porcine mycoplasmas that have a respiratory tropism. Moreover, the overall efficacy of serological detection methods, such as enzyme-linked immunosorbent assays (ELISAs), is often hampered because of antigenic cross-reactions that exist between *M. hyopneumoniae*, *M. flocculare*, and *M. hyorhinis* (2, 18). The *M. hyopneumoniae* genome codes for several immunodominant proteins, among which are the P36 cystolic protein; the P46, P65, and P74 membranous proteins; and the P97 adhesin. These proteins are known to trigger early specific antibody responses in postweaning and growing pigs following acute or initial infection with *M. hyopneumoniae* (11, 14, 19). The corresponding open reading frames (ORFs) are 1,260 bp for P46 surface lipoprotein and 1,803 bp for P65 lipid-modified amphiphilic surface protein. Sequence analysis of P45- and P65-encoding genes revealed the presence of, respectively, three and one translation termination or nonsense UGA codons, which are exceptionally used for tryptophan residues, in addition to TGG in several mycoplasma genes (14).

The indirect immunofluorescence (IIF) assay is still widely used for diagnosis of *M. hyopneumoniae* since it is a rapid and convenient technique for detection of specific antigens in lung tissues. However, in frozen tissue sections, microstructures are most frequently broken and difficult to recognize, and the use of polyclonal antisera may result in nonspecific detection of other pathogens, namely, *M. flocculare* and *M. hyorhinis*. On

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the other hand, the use of MAb s increases the specificity of serological and immunohistochemical antigen detection tests (1, 18, 23).

This paper describes site-directed mutagenesis of TGA codons of the P46 and P65 genes into TGG codons by overlapping PCRs. The modified P46-encoding gene, as well as the C-terminal portion of the modified P65 encoding gene, was cloned in a prokaryotic plasmid vector to allow expression of the entire P46 and N-terminally truncated P65, membranous lipoproteins, in genetically transformed Escherichia coli cells, as recombinant fusion proteins with glutathione S-transferase (GST). The production and characterization of specific anti-P46 and anti-P65, MAb s are also described, as well as their potential application for the specific immunodetec-tion of M. hyopneumoniae authentic membranous proteins by IIF and streptavidin-biotin immunoperoxidase assays using frozen or paraffin-embedded lung sections, respectively. The immunogen- nicity of the recombinant fusion proteins was also investigated in pigs. (This report was taken in part from a dissertation to be submitted by K. Cheikh Saad Bouh to the INRS-Institut Armand-Frappier, in partial fulfillment of the requirements for the Ph.D. degree.)

MATERIALS AND METHODS

Microorganisms and growth conditions. The ATCC 25934 strain of M. hyopneumoniae was obtained from the American Type Culture Collection (ATCC), Manassas, Va., and used as the reference strain in this study. Other mycoplasma strains including the reference ATCC 25095 and J strains of M. hyopneumoniae, M. flocculare (ATCC 27399), Mycoplasma arginini (ATCC 23838), M. hyorhinis (ATCC 17981), and Acholeplasma laidlawii (ATCC 23206) were also obtained at the ATCC and used in comparative antigenic studies. Mycoplasma hyosynovia was kindly obtained from Claude Montpetit, Ministère de l’Agriculture des Pêcheries et de l’Alimentation du Quebec. All available strains were grown in modified Friis medium (12) containing mycoplasma culture-tested free 20% horse serum (Gibco-BRL, New Zealand), 5% fresh yeast extract (Gibco-BRL), methicillin (0.15 mg/ml; Sigma-Alrich, Oakville, Ontario, Canada), bacitracin (0.15 mg/ml; Sigma-Alrich) and thallium acetate (0.08 mg/ml; Sigma-Aldrich). The strains were selected from the DNA sequences of the ATCC 25934 strain (GenBank accession no. D16682 and U50209, respectively). Sequences of the forward primer, P46Sal1, and those of the reverse primers, P46Sal1 and R2SP65, were 5’-ACCGGAATTCCATGAAAGAAAAATGCTTAGAAAAATTTTTTTTTTTTTC-3’, respectively, and those of the reverse primers, P65Sal1 and R2SP65, were 5’-CCGGCGGATCCATGGAGGAGGGAAGTTAAAATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
previously described (24). Membranes were blocked for 2 h in PBS buffer (pH 7.4) containing 0.5% Tween 80, 5% goat serum, and 3% skim milk and cut into 2- to 3-mm-wide strips. Each strip was incubated for 1 h at room temperature in the blocking buffer. The immune reactions were revealed following a 30-min incubation into a 1:2,000 dilution of porcine hyperimmune serum, followed by a final incubation period in the enzyme substrate solution, as previously described (7).

**Immunogenicity of E. coli-expressed proteins in pigs.** In vivo experiments in pigs were approved by the INRS-IAD Animal Care Committee under the supervision of the Canadian Association for Laboratory Animals. The animals used were eight 4- to 5-week-old specific-pathogen-free (SPF) pigs obtained from a breeding farm located in Southern Quebec, Canada. The breeding stock and piglets were tested and proven to be seronegative for porcine reproductive and respiratory syndrome virus, encephalomyocarditis virus, porcine parvovirus, haemagglutinating encephalomyelitis virus, transmissible gastroenteritis virus, and *M. hyopneumoniae*. They were found to be seronegative to *M. hyopneumoniae* by the routinely used commercial block-ELISA (Dako) for detection of antibodies to a P74 membranous protein and were inoculated intramuscularly with a virulent IAF-DM9827 field strain of *M. hyopneumoniae* corresponding to an infectious dose of 10^7 color-changing units/ml. Both groups of pigs were allocated to separate rooms in facilities equipped with a microorganism-free air system. The animals were monitored and out-breeding attempts in modified Friis medium. Axenically collected and processed for histopathology, PCR, and cultivation attempts in modified Friis medium.

**Microscopic analysis of tissue sections.** Thin sections (5 μm thick) of formalin-fixed, paraflin-embedded tissues from the lungs of experimentally infected pigs were routinely processed for hematoxylin-eosin staining, as previously described (7).

**IF staining.** Thin frozen lung sections with typical lesions of enzootic pneumonia were mounted on glass slides and fixed with 100% ice-cold acetone. Once the slides were dried, they were incubated 1 h at 37°C with 100 μl of anti-P46 or anti-P65 MAbs at a dilution of 1:100, washed in PBS, and reacted similarly with fluorescein-conjugated goat anti-mouse IgA-IgG-IgM (heavy and light chain) (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) diluted 1:50 in PBS. After a further washing step, the fluorescent reaction was observed under a UV microscope (Leica, Leitz Wetzlar, Germany).

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**TABLE 1. Primers used for p46 and p65, mutagenesis**

| Mutagenesis and primer | Sequence |
|------------------------|----------|
| p46                    |          |
| P46BamH1               | 5’-ACCGGATCCATGAAAATGTGCTTAGGAAAAATT-3’ |
| P46SalI                | 5’-GGATCCGACCTTAGGATCCAGGATTATCAAC-3’ |
| Kmut46R1a              | 5’-GATAATCTCTTGATGATGTTGCCAAA-3’ |
| Kmut46R2b              | 5’-TTGGGCACCTATCCCTGAGGATTATC-3’ |
| Kmut46F1b              | 5’-GCAAATAATATTGCTACTGCAA-3’ |
| Kmut46R2c              | 5’-TTGCTGACTGAGCCATTTTGTGC-3’ |
| Kmut46F1c              | 5’-TATGTCGCCAGATGGAATTATGAACT-3’ |
| Kmut46R2c              | 5’-AGTTCCATAATTCCATGGGACATA-3’ |
| p65                    |          |
| 65mutIF2(AG)           | 5’-CCGGAATCCATGTTAAATGGCCTGTTAAGAAAAA-3’ |
| R2Sp65                 | 5’-GGGCCGTCGACTTAAATCTGCGATG-3’ |

* The diagram shows an example of TGA mutagenesis.

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membranous proteins as antigens. The use of GST-coated plates permitted the confirmation of the reactivities of pigs sera against recombinant proteins.

**Experimental infection of SPF pigs.** Six crossbred F1 (Landrace × Yorkshire) castrated SPF pigs (5 to 6 weeks old) were obtained from the aforementioned breeding farm. Experimental inoculation of pigs by the intratracheal route was conducted as described previously (7). The animals were separated in two groups, consisting of two control pigs that received only fresh Friis medium whereas the remaining four pigs received an identical volume (8 ml) of a culture of the virulent IAF-DM9827 field strain of *M. hyopneumoniae*, corresponding to an infectious dose of 10^7 color-changing units/ml. Both groups of pigs were allocated to separate rooms in facilities equipped with a microorganism-free filtered in-flowing and out-flowing air system. The animals were monitored clinically and serologically for a 7-week period and then euthanized. Their lungs were aseptically collected and processed for histopathology, PCR, and cultivation attempts in modified Friis medium.
RESULTS

PCR amplification and directed mutagenesis of TGA codons of the P46 and P65 encoding genes. Based on the reported sequences of the P46 (1,260 bp) and P65 (1,803 bp) membranous protein genes of *M. hyopneumoniae* (strain ATCC 25934), oligonucleotide primers were designed so as to permit amplification of the entire ORFs of the targeted genes. The primer pairs P46BamH1-P46SalI and FSLP65-R2SP65, respectively, were used for their capacity to amplify DNA fragments from the P46 and P65 genes of two other reference strains of *M. hyopneumoniae* (ATCC 25934 and ATCC 25095). Expected PCR amplification products from the P46 and P65 genes were obtained with both heterologous strains. No reactivity was obtained when the PCR was performed with genomic DNA extracted from *M. hyorhinis* and *A. laidlawii* species that commonly infect pigs (Fig. 1). Sequencing analyses confirmed the identity of the two genes.

For several *Mycoplasma* genes, including *M. hyopneumoniae*, TGA codons are translated as tryptophan residues rather than corresponding to translational stop signals as in mammalian and other bacterial cells. For *M. hyopneumoniae*, the ORFs coding for P46 (1,260 bp) and P65 (1,803 bp) possess three and one TGA codons, respectively. Therefore, DNA fragments within both targeted genes of the reference ATCC 25934 strain of *M. hyopneumoniae* were amplified by PCR using overlapping oligonucleotide primers that were specially designed so as to permit replacement of the TGA codons in the DNA fragments (Table 1). The DNA fragments were then appropriately ligated, and the complete modified genes were amplified using primers previously used to amplify the entire P46 and P65 encoding genes. Further sequencing analysis confirmed that the three TGA codons located at positions 208, 301, and 760 of the 1,260-bp P46-encoding gene and at position 631 of the 1,803-bp P65-encoding gene had been replaced by TGG cons.

**Cloning, proaryotic expression, and purification of GST-P46 and GST-P65, recombinant fusion proteins.** The mutated P46 and P65 encoding genes were ligated into the proaryotic pGEX-4T1 vector (Pharmacia) and used to transform competent *E. coli* BL21(DE3) cells to produce recombinant proteins fused to GST. The mutated constructs were cloned in pGEX-4T1 by using restriction sites BamHI and SalI for the P46 gene or EcoRI and SalI for an N-terminally truncated P65 (P65c) gene fragment, and the proaryotic pGEX-4T1 vector was digested by the same combination of restriction enzymes, respectively. Ligation steps were conducted overnight at 14°C. The transformed competent *E. coli* cells were then induced by the addition of IPTG in the culture medium to express the fusion proteins GST-P46 and GST-P65c. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of sonicated lysates of IPTG-induced transformed *E. coli* cells revealed the presence of two additional 72- and 70-kDa protein species. Those proteins species could not be detected either in lysates prepared from noninduced bacteria or in lysates prepared from nontransformed bacteria. The M₄s of both recombinant proteins, as estimated from the electrophoretic migration profiles, corresponded to the M₄s determined from the deduced amino acid sequences of the PCR-amplified P46 entire gene or C-terminal moieties of the P65 protein to which was fused the GST protein (M₄ = 26,000).

Following bulk purification of the induced sonicates on glutathione-Sepharose beads, 1.8 to 2.5 mg of recombinant fusion protein were usually recovered from a culture of 500 ml of IPTG-induced and transformed bacteria (Fig. 2A).

**Species specificity of anti-P46 and anti-P65, MAbs.** From two fusion experiments with spleen cells of hyperimmunized mice, a total of 30 hybridoma cell lines secreting anti-P46 MAbs and 26 hybridoma cell lines secreting anti-P65c MAbs could be established. From these hybridomas, eight secreted anti-P46 MAbs and seven secreted anti-P65c MAbs that reacted intensively against homologous proteins (Fig. 2B). These
FIG. 2. Expression of recombinant P46 and P65c and Western blotting patterns of specific monoclonal antibodies. (A) Expression of the GST-P46 and GST-P65c recombinant fusion proteins in E. coli. Lane 1, IPTG-induced GST recovered from pGEX-4T1-transformed bacteria (GST = 26 kDa); lane 2, purified GST-P46 recombinant fusion protein (72 kDa); lane 3, cleaved recombinant GST-P46 fusion protein (46 kDa); lane 4, purified GST-P65c recombinant fusion protein (70 kDa); lane 5, cleaved GST-P65c recombinant fusion protein (44 kDa); lane 6, noninduced recombinant bacteria; lane M, Rainbow molecular mass markers. (B) Polypeptide specificities of pooled MAbs produced against E. coli-expressed P46 and P65, recombinant proteins. The reactivities of pooled MAbs were tested against the fusion and cleaved recombinant proteins. Lane 1, IPTG-induced pGEX-4T1-transformed bacteria (GST = 26 kDa); lane 2, purified GST-P46 recombinant fusion protein (72 kDa); lane 3, cleaved recombinant GST-P46 fusion protein (46 kDa); lane 4, purified GST-P65c recombinant fusion protein (70 kDa); lane 5, cleaved GST-P65c recombinant fusion protein (44 kDa); lane 6, noninduced recombinant bacteria; lane M, Rainbow molecular weight markers. (C) Reactivities of the anti-P46 and anti-P65, MAbs as determined by Western blotting using whole M. hyopneumoniae lysate as antigenic preparation. The figure illustrates the reactivity patterns as follows: lane 1, polyclonal antiserum to M. hyopneumoniae; lane 2, MAb 7D3-E9 (anti-P46); lane 3, MAb 7D3-C11 (anti-P46); lane 4, MAb 6A4-G9 (anti-P46); lane 5, MAb 4D11-C11 (anti-P65c); lane 6, MAb 1D3-C6 (anti-P65c); lane 7, MAb 4D11-G8 (anti-P65c).
secerting hybridomas were subcloned and either maintained in cultures or used to produce ascitic fluids in pristane-primed mice. MAbs that were secreted from the established hybridomas were tested for their complete polypeptide and species specificity by Western blotting against complete antigenic preparations of three reference strains of _M. hyopneumoniae_ (ATCC 25934, ATCC 25095, and J strains) and that of other porcine mycoplasma species, including _A. laidlawii_. All of the MAbs obtained from established hybridomas reacted very specifically toward the three reference strains of _M. hyopneumoniae_ (Fig. 2C) and showed no reactivity toward other mycoplasma species tested (data not shown). The anti-P65c MAbs were all determined to be of the IgG1 isotype, whereas six of the eight anti-P46 MAbs were found to be IgG1 antibodies and two were more likely of the IgG2a isotype.

**Immunogenicity of E. coli-expressed GST-P46 and GST-P65c recombinant fusion proteins.** By indirect ELISA, using recombinant protein as antigen, seroconversion to _M. hyopneumoniae_ was demonstrated for sera collected from pigs which had received one injection with GST-P46 or GST-P65c or both of these recombinant fusion proteins prepared in a vegetable oil adjuvant. Interestingly, within 14 days after the first injection, specific IgG antibody titers higher than 1:10,000 could be detected by ELISA to both membranous proteins that have been injected individually to the pigs. The sera showed no cross-reactivity against heterologous proteins. On the other hand, when the proteins were injected simultaneously to the pigs, the reactivity to P46 was detected by indirect ELISA 2 weeks (PID 14) earlier than that directed to P65c, for which antibody titers higher than 1:10,000 were not detectable before the second dose of the antigenic preparation was given to the pigs (Table 2). Indeed, when the proteins were injected simultaneously, no reactivity was detected 21 days after first injection of the mixture of both recombinant fusion proteins, and _A_450 values higher than 1.0 were not reached until PID 42 or 2 to 3 weeks following the booster injection. Western blotting experiments conducted with sera collected at PID 42 (dilution, 1:100) also confirmed their reactivities against the authentic proteins of _M. hyopneumoniae_.

**Clinical and pathological findings in experimentally infected pigs.** Weaned pigs that were infected with the Quebec field strain IAF-DM9827 of _M. hyopneumoniae_ did not mani-

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**TABLE 2. Absorbance values (_A_450) of serum (dilution, 1:8,000) from pigs immunized with recombinant proteins**

| Pig inoculated (immunization) | _A_450 determined with recombinant protein on: |
|------------------------------|-----------------------------------------------|
|                              | D0       | PID 14    | PID 21    | PID 42    |
|                              | P46      | P65c      | P46      | P65c      |
| 55 (GST – P46)               | 0        | 0.5       | 0.6      | 1.6       |
| 52 (GST – P46)               | 0        | 0.8       | 0.6      | 1.4       |
| 53 (GST – P65c)              | 0        | 0.5       | 0.5      | 1.0       |
| 57 (GST – P65c)              | 0        | 0.5       | 0.5      | 1.0       |
| 56 (P46+P65c)                | 0        | 0.5       | 0.95     | 2.0       |
| 58 (P46+P65c)                | 0        | 0.5       | 0.9      | 0.9       |
| Control 1                    | 0        | 0         | 0        | 0         |
| Control 2                    | 0        | 0         | 0        | 0         |

*Pigs were immunized by injection of individual or mixed P46 or P65c recombinant proteins, and _A_450 was determined by ELISA using either recombinant protein as the antigen.*

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fest clinical signs until 3 to 4 weeks postinoculation. Then, from the 6th to 7th week postinfection, coughing could be elicited by exercising infected pigs around the pen; coughing occurred with greater frequency in the period immediately following the exercise. At this time, the infected pigs were also apatic, reacted less to their environment, and preferred to lay on the ground rather than stand. Compared to the pigs in the control group, the infected pigs also showed a drop in feed consumption, as judged by the quantity of feed remaining in the pens, which were changed twice a day. Rectal temperatures were not taken during this study; body weights and changes in hemograms were also not monitored.

Infected pigs that were necropsied at day 42 or 49 p.i. had gross lesions that were confined to the respiratory tract and thoracic cavity. The lung lesions were confined almost entirely to the apical and cardiac lobes and were clearly demarcated from the normal lung tissue. Plum-colored or greyish areas of consolidation resembling lymphoid tissues were scattered along the ventral borders of the lobes. The mediastinal lymph nodes were enlarged and congested. Minor (25 ml) to large (≥100 ml) amounts of bloody fluid could also be demonstrated within the thoracic cavity and pericardium of two infected pigs. _M. hyopneumoniae_ infection of the lungs and upper respiratory conduct was confirmed by a single P36 PCR assay (6).

**Microscopic lesions and immunohistopathology.** At PID 42, the microscopic lesions observed were confined to the thoracic and cardiac lobes of lung of the infected pigs. The plum-colored and consolidated areas of the infected lungs corresponded to mild to severe characteristic perivascular and peribronchiolar lymphomononuclear nodules of infiltration, often compressing the lumen of the bronchioles. Hyperplasia of the epithelial cells of the affected bronchioles was observed in all four infected pigs, but also, in two pigs the lumen of their bronchioles was completely filled with an infiltrate consisting of cell debris and numerous lymphomononuclear cells. Thin frozen lung sections mounted on glass slides and fixed with 100% ice cold acetone were first tested for the presence of _M. hyopneumoniae_ antigens using pools of either two of three of the anti-P46 or anti-P65 MAbs enriched ascitic fluids, and then reacted with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Boehringer Mannheim) diluted 1:50 in PBS. With both types of MAbs, specific fluorescent cells were usually observed lining the bronchiolar epithelium. The morphology and delimitation of bronchiolar epithelial cells were more easily recognized when slides were incubated with the pool of anti-P46 MAbs (Fig. 3A) compared to the pool of anti-P65 MAbs (Fig. 3B). No such fluorescence was observed when lung sections were incubated with normal mouse serum (Fig. 3C).

On the other hand, immunohistochemical detection of _M. hyopneumoniae_ in lungs of the four experimentally infected pigs could be achieved by immunoperoxidase staining. Both pools of anti-P46 or anti-P65 MAbs and homologous porcine hyperimmune serum, permitted effective detection by indirect immunoperoxidase assay of _M. hyopneumoniae_-specific antigens in formalin-fixed, paraffin-embedded lung sections from pigs that were killed after the 6- to 7-week observation period. In comparison with the IIF method on frozen lung sections, the morphology of lung tissues was much more preserved, such that positive staining was often associated with the typical histopathological lesions described above. Positive
staining associated with the presence of specific *M. hyopneumoniae* antigens was localized as a diffused brownish labeling in the cytoplasm, but more intensively on the surface, of the epithelial cells of large airways such as bronchioles and bronchi, but no staining could be observed of the endothelial cells of alveolar ducts and alveoli. Positively stained hyperplastic epithelial cells of the thoracic bronchioles was observed, mainly following incubation with anti-P46 MAbs, but in all cases, there was no staining of infiltrated lymphomononuclear cells (Fig. 4C and D). Only debris of epithelial cells appeared to be stained in the exudate presented in the lumen of bronchoalveolar ducts. The staining reaction with anti-P65c MAbs was more diffused (Fig. 4E and F). Thin sections of lung tissues from control pigs were negative for the presence of specific antigens, as well as lung sections from experimentally infected pigs that had been incubated with negative mouse sera (Fig. 4B).

**DISCUSSION**

*M. hyopneumoniae* is a fastidious microorganism that is difficult to isolate in culture (1) from clinical specimens on a routine basis, and as the only recognized etiological agent of enzootic pneumonia in pigs, it shares some antigenic determinants with less virulent species, such as *M. hyorhinis* and *M. flocculare* (10). Both of these properties have hampered the development of accurate diagnostic tests necessary to the establishment of control epidemiological programs. The inefficacy of inactivated or killed vaccines (bacterins) to prevent pig infection by *M. hyopneumoniae*, as the result of a poor stimulation of the specific mucosal immunity, makes this pig infectious disease a chronic, costly, and uncontrolled pathological entity with a worldwide distribution. Consequently, there is a need for standardized sources of purified proteins carrying most predominant antigenic determinants and of specific antibodies directed against these proteins for the development of species-specific and accurate diagnostic assays for field strain identification and detection of specific circulating and mucosal antibodies.

Membranous lipoproteins, particularly the P46, P65, and P97 proteins, have been demonstrated to carry species-specific antigenic determinants (34), but their association with a protective immune response is still to be investigated (31).
FIG. 4. Histological and immunohistochemical findings as observed in the lungs of experimentally infected SPF pigs. (A) Peribronchiolar and perivascular accumulation of mononuclear cells with a mild hyperplasia of the bronchiolar epithelium (HE staining). (B to F) Immunoperoxidase staining by negative mouse serum (B) or by anti-P46 (C and D) and anti-P65c (E and F) MAbs of paraffin-embedded sections of lungs from experimentally infected pigs revealed staining patterns similar to that observed following immunofluorescence as indicated by arrows. Magnification, ×18 (A, B, E, and F) and ×36 (C and D).
In the present study, the P46 and P65 membranous proteins of *M. hyopneumoniae* could successfully be expressed in *E. coli*, provided that TGA stop codons were replaced with TGG using directed PCR mutagenesis. Moreover, the prokaryotic pGEX-4T-1 expression system has been shown to easily and rapidly produce large quantities of pure proteins (25, 29), and because of the presence of a thrombin protease recognition site downstream of the GST coding sequences, this allows cleavage of the desired protein from the fusion partner. Different incubation temperatures and concentrations of IPTG used for the induction can be tested to increase the level of production of the recombinant protein and to avoid its accumulation in the form of inclusion bodies (25). Another advantage of this expression system is that GST protein is not present in *E. coli*; hence, pig sera should not possess any antibodies that would react against this protein and interfere with data obtained from serological tests.

The *E. coli*-expressed recombinant proteins displayed the antigenicity of the authentic proteins being recognized by convalescent pig sera by Western blotting and ELISA. The immunogenicity of both authentic proteins was preserved since following injection of mice and SPF pigs, both species produced antibodies that specifically reacted to the authentic P46 and P65 proteins by Western blotting. Furthermore, MAbs generated following fusion experiments with mice hyperimmunized against the recombinant proteins also reacted specifically to the authentic proteins of reference and field isolates of *M. hyopneumoniae*, but not against proteins of other *Mycoplasma* species. Therefore, for diagnosis purpose, the anti-P46 and anti-P65 MAbs could be used for the final identification of *M. hyopneumoniae* field strains isolated in culture.

IIF on frozen tissue sections is probably the most common diagnostic tool used for the detection of *M. hyopneumoniae* in tissues of infected pigs (23, 30; Feenstra et al., Proc. 13th Int. Pig Vet. Soc. Congr.), but the streptavidin-biotin immunoperoxidase techniques have many advantages compared to cultivation methods; they are rapid and sensitive antigen detection tests, and contrary to IIF, they permit the simultaneous quantification of damages or lesions caused by *M. hyopneumoniae* in the lungs tissues and upper respiratory tract airways. However, polyclonal antibodies are still currently used in the IIF and immunoperoxidase tests (1, 22, 23, 30; Feenstra et al., Proc. 13th Int. Pig Vet. Soc. Congr.). Consequently, false-positive results may arise due to cross-reactions that exist between pathogenic (*M. hyopneumoniae* and *M. hyosynoviae*) and non- or less virulent (*M. flocculare* and *M. hyorhinis*) mycoplasma species (10). Therefore, to eliminate misinterpretation due to nonspecific immunolabeling, the use of MAbs which react to specific immunodominant proteins of *M. hyopneumoniae* is suggested. Recently, we demonstrated that MAbs raised against the species-specific P36 cytotoxic protein, and its encoding gene, may be considered for early and specific diagnosis of *M. hyopneumoniae* infection by PCR and IIF on frozen lung sections (6, 7). However, no characteristic immunolabeling pattern could really be defined using anti-P36 MAbs. Herein, anti-P46 and anti-P65 MAbs could be applied for the specific diagnosis of *M. hyopneumoniae* infection by IIF on frozen lung sections and by indirect immunoperoxidase on formalin-fixed, paraffin-embedded lung sections from pigs experimentally infected with a virulent field strain of *M. hyopneumoniae*. The great advantage of the immunoperoxidase labeling technique is that one can easily interpret the pathological lesions in term of cells infected by the microorganism and type of inflammatory cells involved, since the morphology of lung tissues is well preserved and the counterstaining method allows histopathological diagnosis. Positive staining associated with the presence of specific *M. hyopneumoniae* antigens showed that the infection was mainly localized on the surface of epithelial cells of the bronchi and bronchioles. The immune response involved infiltration of the surrounding interstitial tissue by lymphomononuclear cells, which are noninfected by this virulent agent. *M. hyopneumoniae* has not been reported as a tissue invader, but rather it is considered to be an extracellular pathogen which associates very intimately with the ciliated epithelial cells of the porcine lower respiratory tract (22, 26), which is in agreement with the immunofluorescence and immunoperoxidase patterns obtained in the present study. We are currently evaluating the anti-P46 and anti-P65, MAbs for their potential use in a specific and sensitive blocking ELISA for detection of antibodies in pigs.

**ACKNOWLEDGMENTS**

We thank L. Wilson and N. Sawyer for their excellent technical assistance. Particular thanks go to C.A. Gagnon, INRS-Institut Armand-Frappier, for his help in experimental infections and necropsies. We also thank D. Larochelle, Laboratoire de pathologie animale, Ministère de l’Agriculture, des Pêcheries et de l’Alimentation du Québec, Ste-Foy, Québec, Canada, for the preparation of histologic sections.

This work was partly supported by the Conseil de Recherches en Pêche et Agro-Alimentaire du Québec (grant 4600); the Quebec Federation of Swine Producers; and Biovet Inc., St-Hyacinthe, Quebec, Canada.

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