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Molecular Characterization of a Protective Outer Membrane Lipoprotein (OmlA) from *Actinobacillus pleuropneumoniae* Serotype 1†

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An expression library was constructed from an *Actinobacillus pleuropneumoniae* serotype 1 clinical isolate using a plasmid vector. The library was screened with serum raised against the culture supernatant of this strain. One *Escherichia coli* transformant which also reacted with convalescent serum was isolated and found to express a protein with an electrophoretic mobility of approximately 50,000. The *A. pleuropneumoniae*-derived DNA encoding the protein was localized and characterized by nucleotide sequence analysis and primer extension mapping. One open reading frame of 1,095 bases was detected and confirmed by Tn*phoA* insertion mutagenesis. It encoded a protein with a calculated molecular mass of 40 kDa which was lipid modified and present in the outer membrane and in membrane blebs of *A. pleuropneumoniae*. This protein was designated as outer membrane lipoprotein A (OmlA), and the encoding gene as *omlA*. Southern blotting under low-stringency conditions revealed the presence of hybridizing sequences in all *A. pleuropneumoniae* type strains, and a specific serum detected a homologous protein in serotypes 2, 8, 9, 11, and 12 type strains. Pigs immunized with this recombinant protein preparation were protected from death in an aerosol challenge experiment with an *A. pleuropneumoniae* serotype 1 isolate.

Actinobacillus pleuropneumoniae is the cause of a highly infectious porcine pleuropneumonia, a disease which is encountered worldwide (35). Infected pigs develop disease symptoms ranging from acute fibrinous pneumonia and pleuritis with high mortality to chronic lung lesions resulting in reduced growth rates (36), and signs of *A. pleuropneumoniae* disease can be induced in pigs by exposure to cell-free culture supernatant (31). Pigs which survive an infection develop a protective immune response but can still be carriers of the pathogen (36). In protection, the antibody response appears to play an important role. Thus, nonimmune pigs can be passively immunized with porcine antiserum to live bacteria (17).

Attempts to immunize against *A. pleuropneumoniae* disease have been hampered by the occurrence of 12 different serotypes (9, 23–27) and the lack of reliable cross-protection (22). Thus, intramuscular injection of a bacterin prepared from one serotype protects from homologous but not heterologous challenge (14). This antigenic variability among *A. pleuropneumoniae* serotypes has been confirmed by comparison of the outer membrane profiles and by multilocus enzyme electrophoresis indicating a clonal origin of isolates belonging to the same serotype (21).

On the basis of these findings, we hypothesized that a cross-protective immune response might be achievable by immunization with a mixture of serotype-specific antigens that are present in the culture supernatant and that are recognized by convalescent serum. Recently, we have been able to support this hypothesis. Thus, we were able to show that two distinct recombinant antigens could individually protect pigs from death in an *A. pleuropneumoniae* serotype 7 challenge (32). One of these antigens was a truncated RTX-toxin (2, 32), and the other one was an iron-regulated transferrin-binding protein which was also present in cell-free culture supernatant in the form of membrane blebs (8). However, no cross-protection against *A. pleuropneumoniae* serotype 1 infection was observed (32).

Therefore, we further exploited this hypothesis. As described herein, we succeeded in cloning and expressing a protein from *A. pleuropneumoniae* serotype 1 which is present in the culture supernatant and reacts with convalescent serum. We describe the characterization of this protein and investigate its protective efficacy as well as its distribution among the *A. pleuropneumoniae* type strains.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and recombinant plasmid constructs used in this study are described in Table 1. *A. pleuropneumoniae* strains were grown on PPLO medium (Difco Laboratories, Detroit, Mich.) supplemented with β-NAD (10 mg/liter; Sigma Chemical Co., St. Louis, Mo.). Iron restriction was obtained by adding 2,2'-dipyridyl (Sigma Chemical Co.) to a final concentration of 100 μM. *Escherichia coli* transformants were grown in Luria medium (19) supplemented with ampicillin (100 μg/ml). Transcription from the *tac* promoter was induced by the addition of isopropylthiogalactopyranoside (IPTG; 1 mM, final concentration).

Preparation of DNA and Southern blotting. Genomic DNA was prepared by sodium dodecyl sulfate (SDS)-facilitated freeze-thaw-induced lysis as described previously (37). Plasmid DNA was prepared from chloramphenicol (100 μg/ml)-amplified cultures by alkaline lysis and cesium chloride-ethidium bromide gradient centrifugation (19).

All restriction endonuclease digests were performed in T4

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and characteristics
E. coli	
HB101	*hsdS20* (r_B^- m_B^-) *supE44 recA13*
JM105	*hsdR4* Δ(*lac-pro*) [F' *lacI*^{q} ZΔM15]
CC118	*recA1 phoA*Δ*20* λ sensitive (20)
A. pleuropneumoniae	
AP205	*A. pleuropneumoniae* serotype 7 porcine lung isolate provided by M. L. Chepok, Modern Veterinary Products, Omaha, Nebr.
AP37	*A. pleuropneumoniae* serotype 1 porcine lung isolate obtained from the Western College of Veterinary Medicine, Saskatoon, Saskatchewan, Canada
ATCC 27088	Type strain serotype 1
ATCC 27089	Type strain serotype 2
ATCC 27090	Type strain serotype 3
ATCC 33378	Type strain serotype 4
ATCC 33377	Type strain serotype 5A
L20	Type strain serotype 5B provided by R. Nielsen, State Veterinary Serumlaboratory, Copenhagen, Denmark
ATCC 33590	Type strain serotype 6
WF83	Type strain serotype 7 provided by S. Rosendahl, University of Guelph, Ontario, Canada
405	Type strain serotype 8
CVJ13261	Type strain serotype 9] provided by R. Nielsen,
D13039	Type strain serotype 10] State Veterinary
56153	Type strain serotype 11] Serumlaboratory
8329	Type strain serotype 12] Copenhagen, Denmark
Plasmids	
pGH432, pGH433	*lac* repressor and β-lactamase-encoding pBR derivative with *tac* promoter followed by leader peptide with unique *Bam*HI, *Bgl*II, *Sma*I sites for the construction of protein fusions, and stop codons in all three reading frames (2)
pOM37/E1	Encoding the OmlA protein from *A. pleuropneumoniae* AP37
pOM37/E2	*Sty*I deletion derivative of pOM37/E1 encoding the full-length OmlA protein from *A. pleuropneumoniae* AP37
pOM37/E16	*Eco*RV-*Sty*I deletion derivative of pOM37/E1 encoding the full-length OmlA protein from *A. pleuropneumoniae* AP37
pOM37/E17	*Kpn*I-*Sty*I deletion derivative of pOM37/E1 encoding a truncated OmlA protein from *A. pleuropneumoniae* AP37

DNA polymerase buffer (19) supplemented with 1 mM dithiothreitol and 3 mM spermidine. Digested DNA was separated on 0.7% agarose gels and transferred onto nitrocellulose by capillary blotting. ^{32}P-labelled probes were prepared by random priming (7), and unincorporated nucleotides were removed by passage through a Sephadex G-50 column. Filters were prehybridized in 5× Denhardt's solution–6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 8])–0.5% SDS at 65°C. Filters were hybridized in the same solution at 55°C and washed at 55°C in 3× SSC–0.5% SDS (low stringency) or at 65°C in 0.1× SSC–0.5% SDS (high stringency).

Preparation and analysis of inclusion bodies (aggregates), culture supernatants, and membranes. Protein aggregates were prepared as previously described (8). The protein aggregates were judged to be about 70% pure as assessed by SDS-polyacrylamide gel electrophoresis (PAGE) (18) and staining with Coomassie blue. Prior to the immunization of pigs (see below), the concentration of OmlA protein in the aggregate preparations was estimated by separating serial dilutions of the protein by SDS-PAGE and staining with Coomassie blue. The intensity of the bands was compared to those of a bovine serum albumin standard (Pierce Chemical Co., Rockford, Ill.).

Low-speed culture supernatants were obtained by pelleting *A. pleuropneumoniae* cells grown to the mid-log phase at 4,000 × *g* for 20 min and subsequently filtering with a 0.25-μm-pore-size filter. Recentrifugation of this preparation at 100,000 × *g* for 30 min resulted in a pellet consisting of membrane blebs as confirmed by electron microscopy (data not shown) and a bleb-free culture supernatant. Proteins in these low- and high-speed supernatants were recovered by precipitation with 10% trichloroacetic acid (vol/vol) for 1 h at 4°C and subsequent centrifugation.

Total membranes were prepared by French press treatment and subsequent centrifugation at 100,000 × *g* (11). Outer membranes were isolated by sucrose gradient centrifugation with a two-step gradient (10) and by sarcosyl solubilization (4).

Preparation of antisera. Cell-free *A. pleuropneumoniae* serotype 1 low-speed culture supernatant was precipitated with 10% trichloroacetic acid (vol/vol), emulsified with incomplete Freund's adjuvant, and used to immunize rabbits twice, at three-week intervals. Porcine convalescent sera were obtained from pigs experimentally infected by aerosol with approximately 10% of 1 50% lethal dose (LD_{50}) of *A. pleuropneumoniae* serotype 1 strain AP37. Serum against the recombinant OmlA protein was obtained from pigs immunized as described below for the challenge experiment.

Western blotting (immunoblotting). Whole-cell lysates of the *A. pleuropneumoniae* type strains grown in broth under iron-restricted conditions as well as various membrane preparations and culture supernatants were separated by SDS-PAGE and electroblotted onto nitrocellulose (39). Unspe-

cific binding was blocked by incubation in 0.5% gelatin in washing buffer (150 mM NaCl, 30 mM Tris-HCl [pH 8], 0.05% Triton X-100). Subsequently, porcine serum raised against the recombinant OmlA protein and alkaline phosphatase-labelled goat anti-pig conjugate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.), both in washing buffer, were added, and each was incubated for 1 h at room temperature. The blots were developed with a substrate containing Nitro Blue Tetrazolium (100 μg/ml, final concentration) and 5-bromo-4-chloro-3-indolylphosphate (50 μg/ml, final concentration).

Intrinsic radiolabelling with [^{3}H]palmitic acid, immunoprecipitation, and globomycin treatment. Labelling was done as described by Ichihara et al. (16). Briefly, [9,10-^{3}H]palmitic acid (55 Ci/mM) in toluene (Amersham Corp., Arlington Heights, Ill.) was lyophilized and dissolved in isopropanol to a concentration of 5 mCi/ml. Globomycin (a gift from M. Arai, Sankyo Co., Tokyo, Japan) was dissolved in 50% dimethyl sulfoxide at a concentration of 10 mg/ml and used at a final concentration of 100 μg/ml. *A. pleuropneumoniae* AP37 (in PPLO broth) and *E. coli* transformants (in Luria broth containing 1 μM IPTG) were grown with shaking to an optical density at 660 nm of 0.4. Then palmitic acid at 10 μl/ml was added alone or in combination with globomycin, and growth was continued for 1 h. The cultures were precipitated with 10% (vol/vol) TCA, the pellets were washed with methanol, and an immunoprecipitation analysis was performed essentially as described by Huang et al. (15). Since the OmlA-specific serum was obtained from immunized pigs, protein G-Sepharose was used instead of protein A-Sepharose to recover the OmlA-porcine antibody complexes. The immunoprecipitated proteins were resuspended in SDS sample buffer, heated to 80°C for 5 min, and separated by SDS-PAGE. The gels were fixed, treated with Amplify (Amersham Corp.), dried, and exposed to X-ray film. Alternatively, to identify the protein precursors produced by globomycin treatment, cells were pelleted, resuspended in sample buffer, and analyzed by SDS-PAGE and Western blotting with the OmlA-specific serum.

Preparation and screening of the *A. pleuropneumoniae* serotype 1 expression library. Genomic DNA from *A. pleuropneumoniae* AP37 was partially digested with the restriction endonuclease *Sau*3AI. Fragments of 3 to 8 kb were isolated by sucrose gradient centrifugation (19) and ligated into the *Bam*HI and *Bgl*II sites of the expression vectors pGH432 and pGH433, thus allowing for fusions in all three reading frames. *E. coli* HB101 was transformed, plated at a density of approximately 400 colonies per plate, and screened by immunoblot (33). Briefly, colonies were replica plated onto nitrocellulose disks, induced for 2 h with 1 mM IPTG, and lysed in chloroform vapor. Nonspecific binding was blocked with 0.5% gelatin in washing buffer, and after removal of the cellular debris, the membranes were incubated with rabbit serum raised against the *A. pleuropneumoniae* AP37 culture supernatant and developed by using goat anti-rabbit conjugate and substrate as described above.

Transposon Tn*phoA* mutagenesis. Fusions of *omlA* to *phoA* were constructed by using λ::Tn*phoA* (20). In this system, alkaline phosphatase-positive colonies are obtained only if Tn*phoA* transposes into a DNA sequence in such a way that PhoA is fused in frame to an existing open reading frame (ORF) which possesses a signal peptide. Briefly, the phosphatase-negative *E. coli* CC118 was transformed with pOM37/E16, infected with λ::Tn*phoA*, and grown for 18 h at 30°C. Subsequently, aliquots were plated on Luria agar supplemented with ampicillin (100 μg/ml), kanamycin (300 μg/ml), and 5-bromo-4-chloro-3-indolylphosphate (40 μg/ml) and incubated for 2 to 3 days at 30°C. Alkaline phosphatase-positive colonies were pooled, and plasmid was extracted and used to retransform *E. coli* CC118. Plasmids from alkaline phosphatase-positive colonies obtained in this transformation were mapped by restriction enzyme analysis, and the nucleotide sequence at the insertion site was determined by using an oligonucleotide primer complementary to the first 20 bases of the *phoA* gene in Tn*phoA* (3, 20).

Nucleotide sequence analysis. DNA sequencing was performed by using M13 vectors and the dideoxy-chain termination method essentially as described by Sanger et al. (34). Nested deletions were prepared by exonuclease III treatment (13), and specific primers were synthesized with the Pharmacia Gene Assembler. Both strands were sequenced in their entirety. The ORF of the *omlA* gene was confirmed by Tn*phoA* insertion mutagenesis. The sequence was analyzed by using the IBI/Pustell program and the GenBank data base.

Nucleotide sequence accession number. The GenBank nucleotide sequence data base accession number for the *A. pleuropneumoniae omlA* gene is L06318.

Primer extension mapping. RNA was prepared from *A. pleuropneumoniae* AP37 essentially as described by Emory and Belasco (6). Briefly, 25 ml of bacterial culture with an optical density at 660 nm of 0.4 was cooled on crushed ice and centrifuged. The bacterial pellet was resuspended in 250 μl of 10% sucrose–10 mM sodium acetate (pH 4.5) and frozen at −70°C. The pellet was thawed by mixing with an equal volume of hot (70°C) 2% SDS–10 mM sodium acetate (pH 4.5). Then, 375 μl of hot (70°C) H_2O-equilibrated phenol was added, and the tubes were vortexed, frozen at −70°C, and spun for 10 min in an Eppendorf centrifuge. The clear supernatant was removed, 2.5 volumes of ethanol were added, and the RNA was stored at −70°C until needed. The primer extension was done as described previously (38), using a primer complementary to the bases 37 to 57 of the *omlA* ORF. 7-Deaza-dGTP and avian myeloblastosis virus reverse transcriptase were used to prevent compressions.

Immunization and challenge experiment. Fourteen outbred pigs of either sex from an *A. pleuropneumoniae*-free herd approximately 6 weeks of age were randomly assigned to two groups and housed indoors, with controlled temperature and ventilation, on vinyl-covered metal flooring with free access to water and commercially prepared feed. They were cared for in accordance with the principles outlined in the "Guide to the Care and Use of Experimental Animals" of the Canadian Council on Animal Care. Pigs were immunized twice in the neck muscle with 2-ml doses at a 3-week interval. Each dose contained 25 μg of OmlA antigen dissolved in 25 μl of 3.5 M guanidine hydrochloride, 0.66 ml of the adjuvant Emulsigen Plus (MVP Laboratories, Ralston, Nebr.) and isotonic NaCl. Pigs in the control group were injected accordingly with an OmlA-free preparation. Ten days after the second immunization, all pigs were challenged by aerosol in a Plexiglas chamber for 10 min (28). The challenge was prepared by growing *A. pleuropneumoniae* serotype 1 strain AP37 for 4 h at 37°C with shaking to an optical density at 660 nm of 0.8. Prior to aerosolization, the culture was diluted 1:500 in isotonic NaCl, resulting in a living cell count of 6×10^5 ml^{-1} (approximately 5 LD_{50} in this challenge model). The clinical signs of disease in pigs were monitored daily for the first 3 days postchallenge as described previously (32). Pigs that were assessed as having a severely increased respiratory rate and effort and appeared

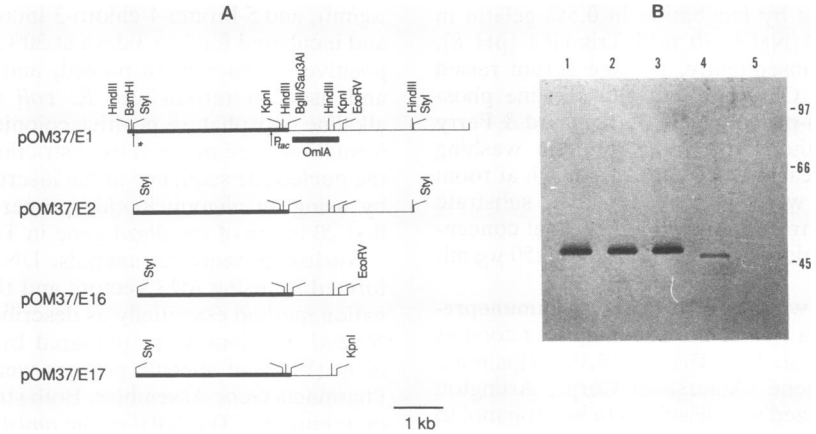

FIG. 1. Physical map of plasmid pOM37/E1 and deletion derivatives and the translational activity of the respective *E. coli* transformants. (A) Vector sequences are represented by the thick lines, and *A. pleuropneumoniae* AP37-derived sequences are shown by the thin lines. The vertical arrow labelled P_{tac} indicates the position of the *tac* promoter, and the asterisk-labelled arrow indicates the location of stop codons in all three reading frames. The OmlA-labelled bar indicates the location of the ORF as determined by the Western blots of deletion derivatives. (B) Western blot of whole-cell lysates from *E. coli* transformants containing pOM37/E1 (lane 1), pOM37/E2 (lane 2), pOM37/E16 (lane 3), pOM37/E17 (lane 4), and pGH432 (lane 5). *E. coli* transformants were not IPTG induced, and the blot was developed with serum from OmlA-immunized pigs. Numbers on right show size in kilodaltons.

to be lethargic were humanely killed. The trial was terminated 1 week after challenge, and all lungs were cultured.

The serum titers of pigs before and after immunization were assessed by enzyme-linked immunosorbent assay (ELISA) with the recombinant OmlA protein as the coating antigen. Briefly, Immulon 2 plates (Dynatech Laboratories, Inc., Chantilly, Va.) were coated with 100 μl of OmlA antigen (1 μg/ml in carbonate buffer) at 4°C for 16 h. Plates were washed with distilled water before the addition of serum, conjugate, and chromogen. Sera were diluted 1:100 initially and further twofold in the plates in BLOTTO diluent (150 mM phosphate-buffered saline [pH 7.2], 50 g of powdered instant milk per liter, 100 μl of antifoam B [Sigma Chemical Co.] per liter, 1 g of thimerosal per liter). A standard positive serum with a titer of 12,500 was used on each plate. Serum dilutions and goat anti-pig alkaline phosphatase conjugate were each incubated for 1 h at room temperature. Chromogen consisted of 100 μl of *p*-nitrophenyl phosphate (3 mg/ml) in 1 M diethanolamine (pH 9.8)–0.5 mM $MgCl_2$. Plates were developed for 30 min at 37°C and read at 405 nm.

RESULTS

Cloning and expression of *A. pleuropneumoniae* AP37 *omlA* gene. Approximately 6,000 transformants, after IPTG induction, were screened in a colony immunoblot with serum raised against the culture supernatant. Eight immunoreactive colonies were isolated. They were induced with IPTG and examined in a Western blot with porcine convalescent serum. One protein with an electrophoretic mobility of 50,000 comigrated with an immunoreactive protein from *A. pleuropneumoniae* serotype 1, and it reacted with both serum raised against the culture supernatant and convalescent serum (data not shown). The *E. coli* transformant was found to produce small amounts of this protein without induction. Upon induction with IPTG, the protein was present in large amounts in the form of inclusion bodies. The antigen was designated as OmlA (outer membrane lipoprotein A), and the encoding plasmid as pOM37/E1 (Fig. 1). Physical mapping showed that the plasmid contained a 5-kb insert. Several deletion derivatives were constructed, and it was observed that transformants containing the deletion derivative pOM37/E17 produced a truncated protein (Fig. 1), thus indicating that the encoding gene overlaps the *Kpn*I restriction enzyme site.

Analysis of *A. pleuropneumoniae* AP37-derived *omlA* gene and encoded protein. The nucleotide sequence analysis of pOM37/E16 revealed one ORF of 1,083 bp coding for a protein with a predicted molecular mass of 39,780 Da (Fig. 2). It was preceded by a Shine-Dalgarno consensus sequence AAGGAA 8 bp upstream of the methionine codon. The first 19 amino acids of the polypeptide have the characteristics of a lipoprotein signal peptide (12) with a predicted cleavage site in front of the cysteine residue at position 20. The ORF was confirmed by two independent Tn*phoA* insertions 50 and 530 bp downstream from the methionine codon which, upon transformation of the *phoA*-negative *E. coli* CC118, gave rise to alkaline phosphatase-positive transformants. A GenBank data base homology search with the predicted amino acid sequence of OmlA did not reveal likely similarities (>35%) to known ORFs or polypeptides.

The primer extension analysis (data not shown) located the beginning of the mRNA at a T residue 76 bp upstream of the methionine start codon. The −10 and −30 regions are both A+T-rich, and the promoter structure matches the *E. coli* consensus characteristics (Fig. 2).

The predicted signal peptide cleavage site resulting in an amino-terminal cysteine residue of the mature protein was confirmed by labelling of the *E. coli* transformants with [^{14}C]palmitate and subsequent immunoprecipitation with porcine anti-OmlA serum (Fig. 3A). In addition, it was shown that growth of *A. pleuropneumoniae* AP37 in the presence of globomycin inhibited the palmitate labelling of OmlA (Fig. 3A) as well as the processing of the OmlA precursor protein (Fig. 3B).

The expression of the OmlA protein was, unlike that of the TfbA protein (8), independent of the level of iron in the growth medium. The protein was present in whole membranes, outer membranes as prepared by sucrose gradient

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    Sau3AI
  1 GATCGGCTTT TACAGCGATT GCAGAATGAT TGAATTGTAA ACTTTAGAGC TTTATATTTT

 61 GTTTAATGGT ATTATATTTA CTTATATTTA TGATTCTTAG TTTTTATTGT AAATTAAAGT
      PS-box      ↳mRNA          SspI
121 GTTTATTTAT TGTATTTTAA GTATAAGGAA TTTTTTAATG AATATTGCAA CAAAATTAAT
                           SD          M  N  I  A  T  K  L  M
                              ┌TnphoA
181 GGCTAGCTTA GTCGCTAGTG TAGTGCTTAC CGCATGTAGT GGCGGCGGCT CATCGGGTTC
     A  S  L  V  A  S  V  V  L  T  A  C  S  G  G  G  S  S  G  S

241 ATCGTCTAAA CCAAATTCGG AACTTACACC TAAGGTTGAT ATGTCCGCAC CAAAAGCGGA
     S  S  K  P  N  S  E  L  T  P  K  V  D  M  S  A  P  K  A  E
                                       BstBI
301 GCAGCCAAAA AAAGAGGAAG TTCCACAAGC GGATAATTCG AAAGCGGAAG AACCAAAAGA
     Q  P  K  K  E  E  V  P  Q  A  D  N  S  K  A  E  E  P  K  E

361 GATGGCTCCG CAAGTAGATA GCCCGAAAGC GGAAGAACCA AAAAATATGG CTCCACAAAT
     M  A  P  Q  V  D  S  P  K  A  E  E  P  K  N  M  A  P  Q  M

421 GGGTAATCCA AAACTAAATG ACCCACAAGT AATGGCTCCG AAAATGGATA ATCCGCAAAA
     G  N  P  K  L  N  D  P  Q  V  M  A  P  K  M  D  N  P  Q  K

481 AGATGCCCCA AAAGGAGAAG AACTAAGTAA GGATAAAAGT AATGCGGAAA TTCTTAAGGA
     D  A  P  K  G  E  E  L  S  K  D  K  S  N  A  E  I  L  K  E

541 ATTAGGGGTT AAGGATATTA ATTCAGGTAT CATTAATAAT GCTGATGTAG TTCTGAATTT
     L  G  V  K  D  I  N  S  G  I  I  N  N  A  D  V  V  L  N  L

601 AAAAATAGAT GAAAAAGATC ACATTACAGT CGTATTAGAT AAGGGTAAGA TTAATCGTAA
     K  I  D  E  K  D  H  I  T  V  V  L  D  K  G  K  I  N  R  N

661 TCATCTAAAA GTAACTAATA CAATTTCTGC TCAAGACATT AAAACCTTAA AAGATTCTTC
     H  L  K  V  T  N  T  I  S  A  Q  D  I  K  T  L  K  D  S  S
                              ┌TnphoA
721 AGGCAAATTG TTGGGTTACT ATGGATATAT GCAGTTAAAT CAAGTTCGAC AAGATGAAAA
     G  K  L  L  G  Y  Y  G  Y  M  Q  L  N  Q  V  R  Q  D  E  N
                                 SspI
781 TTATAGCGAT GAAAAAGTTA GTTTGAATGA ATATTATTTA TTATCAATGA ACGATGCCGA
     Y  S  D  E  K  V  S  L  N  E  Y  Y  L  L  S  M  N  D  A  D

841 TAAAATACGT CCGACTAAAT CTATATCATA TAAGGGAGAC ATGTTTTATA GTTACAAAGA
     K  I  R  P  T  K  S  I  S  Y  K  G  D  M  F  Y  S  Y  K  D
                                    HindIII
901 TGTAGGAAAT CAGAAATTAA AGGCTTCTGT AGAAGCTTCT TATGATGATG TAACAAAAAA
     V  G  N  Q  K  L  K  A  S  V  E  A  S  Y  D  D  V  T  K  K

961 AGTATCAATG AAAGTATTTG GTGAGAATAA TGATTACTGG AAATTAGGTG AGTTTGGTAG
     V  S  M  K  V  F  G  E  N  N  D  Y  W  K  L  G  E  F  G  R
                                                      KpnI
1021 AACTAATTTA TTAGAAAATC AAGTGACTGG AGCAAAAGTT GGCGAAGATG GTACCATTAT
      T  N  L  L  E  N  Q  V  T  G  A  K  V  G  E  D  G  T  I  I

1081 AAATGGAACT TTATATTCTA AAATAGATAA TTTTCCTTTA AAACTAACTC CTGACGCAAA
      N  G  T  L  Y  S  K  I  D  N  F  P  L  K  L  T  P  D  A  N

1141 CTTCTCTGGG GGTATTTTCG GTAAAAATGG CGAAGTATTA GCCGGAAGTG CTATTAGTGA
      F  S  G  G  I  F  G  K  N  G  E  V  L  A  G  S  A  I  S  E

1201 AAAATGGCAA GGCGTAATCG GTGCTACGGC AACCACAAAA GAAGATAAAA AATAAACGCT
      K  W  Q  G  V  I  G  A  T  A  T  T  K  E  D  K  K  *

1261 TTGCTAACTA AACCAAAAGT TATCCTTCGG GATAGCTTTT TTACTTTTTA ATCAGACCTA
                 EcoRV
1321 ATAGTGCATC GGTAAAAGAT ATC
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FIG. 2. Nucleotide sequence of the *A. pleuropneumoniae* AP37-derived DNA in pOM37/E16 and the deduced amino acid sequence of the OmlA protein. PS-box indicates the position of a Pribnow-Schaller box consensus sequence. The T residue at position 82 is the transcriptional start site as determined by primer extension mapping. SD marks the Shine-Dalgarno consensus sequence. The underlined boldfaced C marks the amino-terminal cysteine residue of the mature peptide. The Tn*phoA*-labelled marks indicate the position of two independent Tn*phoA* insertions. The underlined amino acids mark a repetitive region in the deduced protein sequence. The underlined palindrome of nucleotides between positions 1275 and 1300 have the potential to form a loop.

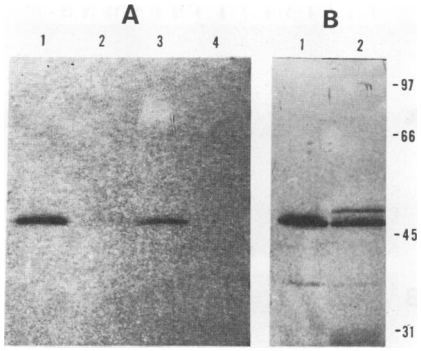

FIG. 3. OmlA protein is lipid modified. (A) Immunoprecipitation of *A. pleuropneumoniae* labelled with [^{14}C]palmitate in the absence (lane 1) or presence (lane 2) of globomycin or of *E. coli* transformed with pOM37/E16 (lane 3) or pGH432 (lane 4). (B) Western blot, with porcine serum raised against the recombinant OmlA protein, of *A. pleuropneumoniae* AP37 grown in the absence (lane 1) and presence (lane 2) of globomycin. The numbers to the right indicate the relative positions of size markers in kilodaltons.

centrifugation, and low-speed membrane bleb-containing culture supernatant; it was absent in sarcosyl-treated outer membranes and in high-speed supernatants (Fig. 4).

Distribution of *omlA* gene and OmlA antigen in *A. pleuropneumoniae* type strains. Genomic DNA from all *A. pleuropneumoniae* type strains was analyzed in a Southern blot with the *Ssp*I fragment of pOM37/E16 which is located within the *omlA* gene as the probe (Fig. 2). The *Sty*I-restricted DNA from all *A. pleuropneumoniae* type strains reacted with the probe under low-stringency conditions (Fig. 5A), and the DNA from serotypes 1, 2, 8, 9, 11, and 12 remained hybridized to the probe under high-stringency washing conditions (Fig. 5B).

Whole-cell lysates from all *A. pleuropneumoniae* type strains, grown under iron-restricted conditions, were analyzed in a Western blot with the serum from pigs immunized with the recombinant OmlA protein (Fig. 6). The same strains that hybridized to the DNA probe under high-stringency washing conditions reacted with the serum raised against the recombinant OmlA protein, and the whole-cell lysates from the *A. pleuropneumoniae* type strains for serotypes 1, 9, and 11 appeared to react more strongly than those of serotypes 2, 8, and 12 (Fig. 6).

Challenge of pigs immunized with recombinant OmlA protein. All pigs immunized with the OmlA protein developed a strong antibody response against the OmlA protein 1 week after the second injection. None of the pigs showed adverse reaction such as abscess formation at the injection site. Upon aerosol challenge with 5 LD_{50} of the homologous *A.*

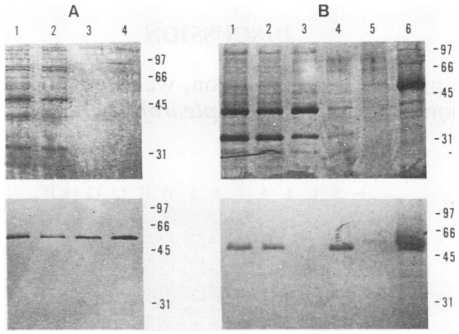

FIG. 4. OmlA protein is not regulated by iron (A) and is associated with the outer membrane (B). (A) Coomassie blue stain (top) and Western blot (bottom) depicting *A. pleuropneumoniae* whole-cell lysates grown under standard (lane 1) and iron-restricted (lane 2) conditions and the corresponding trichloroacetic acid-precipitated supernatants (lanes 3 and 4). (B) Coomassie blue stain (top) and Western blot (bottom) of total membranes (lane 1), outer membranes prepared by sucrose gradient centrifugation (lane 2), outer membranes prepared by sarcosyl extraction (lane 3), membrane blebs (lane 4), supernatant from high-speed centrifugation (lane 5), and aggregate preparation from *E. coli*(pOM37/E16) transformants (lane 6). In all lanes, the material obtained from approximately 250 μl of bacterial culture was loaded. The Western blots were done with porcine serum raised against the recombinant OmlA protein. The numbers to the right indicate the relative positions of size markers in kilodaltons.

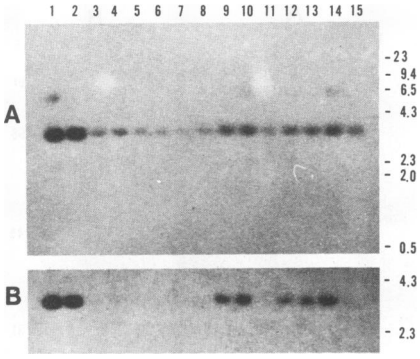

FIG. 5. Occurrence of *omlA* gene-like sequences among the *A. pleuropneumoniae* type strains. The probe used is the *Ssp*I fragment located between positions 161 and 810 of the nucleotide sequence (Fig. 2). The lanes contain *Sty*I-restricted DNA of *A. pleuropneumoniae* type strains for serotypes 1 (lane 1), 2 (lane 2), 3 (lane 3), 4 (lane 4), 5A (lane 5), 5B (lane 6), 6 (lane 7), 7 (lane 8), 8 (lane 9), 9 (lane 10), 10 (lane 11), 11 (lane 12), and 12 (lane 13) and *A. pleuropneumoniae* clinical isolates AP37 (lane 14) and AP205 (lane 15). The top blot was washed at low-stringency conditions; the bottom blot was rewashed at high-stringency conditions. The numbers to the right indicate the relative positions of size markers in kilobases.

pleuropneumoniae serotype 1 strain, pigs in the immunized group showed a significantly lower mortality than pigs in the control group (Table 2). Immunized pigs showed limited clinical signs of disease with a slight to moderate increase in respiratory rate and effort and did not show severe depression. All unimmunized pigs contracted fatal disease or were euthanized in extremis, and *A. pleuropneumoniae* was isolated from all lungs. One week after challenge, the experiment was terminated. The postmortem analysis revealed the presence of moderate lung lesions in all immunized pigs, and *A. pleuropneumoniae* was isolated from all lungs.

DISCUSSION

In the present communication, we report the cloning and expression in *E. coli* of an *A. pleuropneumoniae* serotype 1

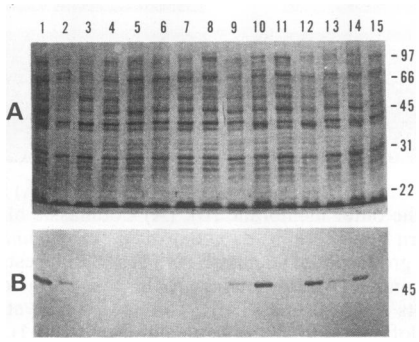

FIG. 6. Distribution of the OmlA protein among the *A. pleuropneumoniae* type strains. Coomassie blue-stained gel (A) and Western blot (B) of whole-cell lysates of *A. pleuropneumoniae* type strains for serotypes 1 (lane 1), 2 (lane 2), 3 (lane 3), 4 (lane 4), 5A (lane 5), 5B (lane 6), 6 (lane 7), 7 (lane 8), 8 (lane 9), 9 (lane 10), 10 (lane 11), 11 (lane 12), and 12 (lane 13) and *A. pleuropneumoniae* clinical isolates AP37 (lane 14) and AP205 (lane 15). The serum used was obtained from pigs immunized with OmlA. Numbers to the right show relative positions in kilodaltons.

TABLE 2. Protective effect of vaccination with OmlA

Treatment	Titer at challenge[a]	Mortality
Vaccinated with OmlA	12,800	1/7
Control	<100	7/7
Statistical significance[b]	<0.001	<0.02

[a] Geometric mean of the ELISA titers to the OmlA protein. The individual values ranged from 3,200 (in the pig that died) to 76,800.

[b] Statistical analysis was done by using two-group *t* test and two-way frequency table programs of BMDP Statistical Software, Inc., Los Angeles, Calif.

polypeptide. *E. coli* transformants carrying the recombinant plasmid pOM37/E1 can be induced by the addition of IPTG to produce inclusion bodies of the OmlA protein, and immunization of pigs with this antigen resulted in a protective immune response. Therefore, we attempted to thoroughly characterize this protective antigen and the encoding gene.

The construction of several deletion derivatives mapped the location of the *omlA* gene, and the smallest plasmid still expressing the full-length protein was designated pOM37/E16 (Fig. 1). The expression of the immunoreactive product in the absence of IPTG suggested that a promoter recognizable by *E. coli* was located on the *A. pleuropneumoniae*-derived DNA upstream of the ORF. The inducibility by IPTG, as well as the truncated polypeptide produced by *E. coli*(pOM37/E17) transformants, indicated the location of the carboxy-terminal end of the *omlA* gene as well as its direction of transcription (Fig. 1).

The nucleotide sequence analysis of the *A. pleuropneumoniae*-derived DNA in pOM37/E16 revealed a single ORF which was confirmed by Tn*phoA* insertion analysis (Fig. 2). One of these insertions was found to be located within the signal peptide. The expression of a functional PhoA protein in this fusion is probably due to its location behind the hydrophobic core of the signal peptide. The transcriptional start site as determined by primer extension analysis (Fig. 3) is preceded by a −10 and −30 region similar to those common in *E. coli* promoters (30), and this finding is in accordance with the expression found in noninduced *E. coli* transformants. Downstream of the ORF, a palindromic sequence of 26 bp is present which might act as a terminator sequence (1).

The ORF encodes a polypeptide with a predicted molecular mass of 39,780 Da. In contrast, a molecular weight of 50,000 had been estimated by SDS-PAGE. This difference could be due to the relatively high content of proline residues in the OmlA protein of 5% (29). Residue 20 of the predicted amino acid sequence is a cysteine, and the first 20 amino acids have the characteristics of a lipoprotein signal peptide (12). The globomycin-specific inhibition of signal peptide cleavage (5) as well as the labelling of the mature OmlA protein with palmitate indicates that the cysteine is lipid modified and represents the first residue of the mature protein. The second residue of the mature protein is a serine which allows the targeting of the protein for the outer membrane (40). The cell fractionation experiments confirmed this location in *A. pleuropneumoniae*. In addition, the absence of the OmlA protein in detergent-treated outer membranes (Fig. 4) and the lack of predicted membrane-spanning regions indicate that the lipid modification serves as a membrane anchor. Last, the absence of a clear OmlA band on the Coomassie blue-stained gels indicates that it is a minor outer membrane protein (Fig. 4).

We observed that *omlA*-like sequences are present in the type strains for all *A. pleuropneumoniae* serotypes (Fig. 5A). However, the hybridization profile with high-stringency washing conditions showed the presence of highly homologous sequences only in the *A. pleuropneumoniae* serotype 1, 2, 8, 9, 11, and 12 type strains (Fig. 5B), and these strains also expressed proteins immunologically similar to OmlA (Fig. 6). Immunization of pigs with the recombinant OmlA protein induced a strong immune response and significantly lowered mortality. However, all immunized pigs developed lung lesions, and *A. pleuropneumoniae* was still present in the lungs 1 week after challenge.

These results demonstrate that protection from *A. pleuropneumoniae* serotype 1-induced mortality can be achieved by immunization with a serotype-specific protein which is present in cell-free culture supernatant and recognized by convalescent serum. However, in accordance with our *A. pleuropneumoniae* serotype 7 challenge experiments (32), immunization with one protein alone does not prevent lung damage or the survival of bacteria in the lung. In conclusion, these results support our hypothesis indicating that a cross-protective *A. pleuropneumoniae* subunit vaccine which effectively prevents mortality and possibly reduces lung damage should be obtainable by combining several recombinant serotype-specific antigens.

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