Molecular Characterization of a Common 48-Kilodalton Outer Membrane Protein of *Actinobacillus pleuropneumoniae*

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Previous studies have shown that a vaccine prepared from outer membranes of *Actinobacillus pleuropneumoniae* serotype 5 can elicit protective immunity in swine against challenge with either serotype 5 or serotype 1. These results suggest the presence of common subcapsular surface antigens, such as outer membrane proteins, that contribute to cross-protective immunity. We have identified a 48-kDa outer membrane protein that is common to all 12 capsular serotypes of *A. pleuropneumoniae* but is not present in the outer membranes of related species of gram-negative swine pathogens. This protein is immunogenic in swine infected with either *A. pleuropneumoniae* serotype 5 or 1A, as well as in swine vaccinated with *A. pleuropneumoniae* serotype 5 outer membranes. This 48-kDa protein is readily detected in outer membranes produced by sucrose density gradient centrifugation, but it is sarcosyl soluble and therefore not found in outer membranes prepared by detergent treatment. The gene encoding the 48-kDa outer membrane protein has been cloned from *A. pleuropneumoniae* serotype 5 and has been designated *aopA*, for *Actinobacillus* outer membrane protein A. The gene is 1,347 bp in length and encodes a protein, designated AopA, of 449 amino acids with a predicted molecular weight of 48,603. Southern blot analysis under high-stringency conditions showed that strains of all 12 serotypes of *A. pleuropneumoniae* contain DNA homologous to this gene, as do strains of two closely related species, *Actinobacillus suis* and *Pasteurella multocida*. Whether antibodies against the AopA antigen contribute to cross-protective immunity against *A. pleuropneumoniae* infection remains to be determined.

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia, a highly contagious and often fatal respiratory disease of swine (5, 10, 21, 32). The disease is characteristically an acute necrotizing hemorrhagic broncho-pneumonia with accompanying fibrinous pleuritis; the clinical course ranges from hyperacute to chronic (21, 32). An acute outbreak or a chronic infection situation can be economically devastating because of mortality, reduced weight gain, poor feed efficiency, and increased medication costs (10). The organism does not spontaneously leave a herd, so once a herd is infected, it remains infected (5). Currently there are no serological tests available for the accurate identification of individual *A. pleuropneumoniae* carriers, so there is no sure way to prevent the introduction of *A. pleuropneumoniae* into a naive herd via purchased animals. Food safety concerns will progressively limit the use of antibiotics in fattening pigs, making immunoprophylaxis even more important. Therefore, the development of both improved vaccines and serological tests remains a major issue.

There are 12 serotypes of *A. pleuropneumoniae* based on antigenic differences in the capsular polysaccharide (25), although only serotypes 1, 3, 5, and 7 are important in the United States (5, 32). Known virulence factors include capsule (12, 42), lipopolysaccharide (LPS) (12, 39, 42), and hemolysin/cytotoxins (6a, 13, 34, 38). A natural or experimental infection with one serotype of *A. pleuropneumoniae* generally provides protection against a subsequent infection with any serotype (22, 23). However, currently used killed whole-cell vaccines, which elicit mainly antibody to capsular polysaccharides, provide only partial protection against infection with the *A. pleuropneumoniae* serotype(s) used in the vaccine and virtually no cross-protection against other serotypes (5, 35). These data suggest the existence of common subcapsular antigens or infection-associated antigens that confer cross-protective immunity and that are exposed during infection and not by vaccination. Isolates of all 12 *A. pleuropneumoniae* serotypes contain several common outer membrane (OM) proteins (OMPs), including a 16- to 18-kDa protein found in many species of gram-negative bacteria (16, 27), a 29/41-kDa heat-modifiable protein, and a major protein that varies from 38 to 42 kDa depending on the serotype (16, 19, 27, 29). Western blot (immunoblot) analysis has demonstrated that convalescent-phase sera from *A. pleuropneumoniae*-infected pigs contain antibodies against these as well as several other common OMPs. We have produced and tested an OM vaccine from *A. pleuropneumoniae* serotype 5 (*A. pleuropneumoniae* 5) which contains OMPs, capsular polysaccharide, and LPS, and we have found that this vaccine provides excellent protection against infection with both the homologous and heterologous serotypes, demonstrating that cross-protective immunity can be achieved by vaccination if the subcapsular antigens are exposed (20).

To identify specific common OMPs of *A. pleuropneumoniae* that could potentially elicit a protective immune response, we have produced monoclonal antibodies (MAbs) against OMs prepared from *A. pleuropneumoniae* 5 (18). Several of these MAbs demonstrated bactericidal activity against *A. pleuropneumoniae* in an in vitro assay (18). We have used these MAbs to identify OMPs that are candidates for a purified protein vaccine against *A. pleuropneumoniae* and to identify recombinant clones expressing these proteins. In this paper, we report on the identification, cloning, and sequencing of a gene

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TABLE 1. *A. pleuropneumoniae* strains used in this study

Strain	Serotype	Source
27088	1A	ATCC[a]
158	1B	V. Rapp, Iowa State University
27089	2	ATCC
27090	3	ATCC
33378	4	ATCC
178	5	V. Rapp, Iowa State University
K17	5A	R. Nielsen, State Veterinary Serumlaboratory, Copenhagen, Denmark
L20	5B	R. Nielsen, Copenhagen, Denmark
33590	6	ATCC
53	7	V. Rapp, Iowa State University
405	8	R. Nielsen, Copenhagen, Denmark
CVJ1326	9	R. Nielsen, Copenhagen, Denmark
13039	10	R. Nielsen, Copenhagen, Denmark
56513	11	R. Nielsen, Copenhagen, Denmark
1096	12	R. Nielsen, Copenhagen, Denmark

[a] ATCC, American Type Culture Collection, Rockville, Md.

encoding a 48-kDa protein that is present in the OMs of all 12 *A. pleuropneumoniae* serotypes.

(A preliminary report of these findings was presented at the 93rd General Meeting of the American Society for Microbiology, Atlanta, Ga., 1993 [2a].)

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *A. pleuropneumoniae* reference strains (Table 1) and field isolates of other gram-negative swine pathogens (received from either NOBL Laboratories, Inc., Sioux Center, Iowa, or the Animal Health Diagnostic Laboratory, Michigan State University, East Lansing) used in this study were grown at 37°C in brain heart infusion medium (Difco Laboratories) supplemented with NAD (10 μg/ml). *Escherichia coli* LE392 (F$^-$ hsdR514 supE44 supF58 lacY1 galK2 galT22 metB1 trpR55 λ$^-$) was used as the host strain for the recombinant λ-Dash library, and *E. coli* Y1090 [lacU169 proA$^+$ Δlon araD139 strA supF trpC22::Tn10(pMC9 Amp^r Tet^r)] was the host for recombinant λgt11. *E. coli* JM105 {supE endA sbcB15 hsdR4 rpsL thi Δ(lac-proAB) F′ [traD36 proAB$^+$ lacI^q Δ(lacZ)M15]} was used as the recipient strain in all transformation and transfection experiments. *E. coli* strains were grown in Luria medium (30), which was supplemented with ampicillin (50 μg/ml) to select plasmid-containing transformants and with isopropyl-β-D-thiogalactoside (IPTG) (1 mM) to induce protein production.

Preparation of outer membranes. Outer membranes were prepared essentially by the procedure of Johnston and Gotschlich (14). Bacteria were grown overnight in the appropriate broth medium in a 37°C water bath shaker. Cells were harvested by low-speed centrifugation, washed with phosphate buffer, and resuspended in a solution of 0.75 M sucrose, 0.01 M Tris-acetate (pH 7.8), and 0.2 mM dithiothreitol. The cells were spheroplasted by adding lysozyme to a concentration of 150 μg/ml. The periplasmic fraction was removed by centrifugation, and the spheroplasts were ruptured by sonication. Cell envelopes were separated from the cytoplasmic fraction by ultracentrifugation for 1 h at 150,000 × g, and the total cell membrane pellet was resolved into cytoplasmic membranes and OMs by isopycnic sucrose density gradient centrifugation (14). In solubilization experiments, the total membrane fraction or the isolated OM was further treated with 1% sarcosyl for 30 min at room temperature; sarcosyl-insoluble membrane components were then pelleted by ultracentrifugation.

Production of antibodies. MAbs against *A. pleuropneumoniae* 5 strain ISU 178 (*A. pleuropneumoniae* 5) OM antigens were produced by standard procedures (24). In brief, spleen cells from BALB/c mice immunized with *A. pleuropneumoniae* 5 OMs were fused with SP2/0 myeloma cells. Antibodies produced by the hybridomas were screened by using an enzyme-linked immunoassay (ELISA) with *A. pleuropneumoniae* 5 OM as the antigen. Antibodies that were positive by ELISA were further characterized by immunoblotting against *A. pleuropneumoniae* 5 OM, as described below. Antibody-containing ascites fluid was produced in pristane-primed BALB/c mice by standard procedures (24).

Convalescent-phase sera were collected at 6 weeks postinfection from pigs experimentally infected with either *A. pleuropneumoniae* 1A or 5 (36). Sera were also collected at 2 weeks after the final vaccination from swine vaccinated three times at 2-week intervals with (i) an *A. pleuropneumoniae* 1A formalinized whole-cell bacterin, (ii) an *A. pleuropneumoniae* 5 formalinized whole-cell bacterin, (iii) an *A. pleuropneumoniae* 5 OM vaccine containing 5 mg of *A. pleuropneumoniae* 5 OM in 25% Emulsigen adjuvant (Modern Vet Products, Ralston, Nebr.) per dose, and (iv) a commercial killed whole-cell bacterin. Convalescent-

phase sera from swine infected with *Actinobacillus suis*, *Haemophilus parasuis*, *E. coli*, and *Pasteurella multocida* were received from Brad Fenwick, Kansas State University.

Immunologic screening of phage libraries. An *A. pleuropneumoniae* 5 genomic library in the phage vector λ-Dash, kindly provided by D. K. Struck, Texas A&M University, College Station, was screened by using a pool of MAbs that were made against *A. pleuropneumoniae* 5 OMs. The library was plated on 90-mm-diameter plates at a density of 500 plaques per plate. Plaques were transferred to nitrocellulose filters, which were probed with the pooled MAbs essentially as described by Huynh et al. (11), with the following modifications. The filters were blocked for 30 min at room temperature with 1% bovine serum albumin (BSA) (radioimmunoassay grade; Sigma Chemical Company, St. Louis, Mo.) in TBST buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05% Tween 20) and then incubated overnight with the pooled MAbs diluted with 1% BSA–TBST and 0.05% sodium azide. After being washed with TBST, the filters were incubated with horseradish peroxidase-labelled goat anti-mouse immunoglobulin G and immunoglobulin M (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) diluted 1:10,000 in 1% BSA–TBST for 1 h at room temperature. The filters were washed in TBST and developed in Tris-buffered saline containing 0.05% 4-chloronaphthol (Bio-Rad Laboratories, Richmond, Calif.), 16.6% methanol, and 0.015% hydrogen peroxide. Positive plaques were picked and rescreened as described above until a homogeneous population of immunopositive plaques was obtained.

Preparation of recombinant proteins from phage and bacteria. To analyze proteins expressed from the bacteriophage clones, plate lysates were prepared directly from the λ-Dash clones and after IPTG induction of the λgt11 clones. The plate lysates were cleared of bacterial debris and soft agar by centrifugation. Proteins were precipitated by addition of an equal volume of 20% ice-cold trichloroacetic acid. The protein pellet was washed with methanol, resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (15), and boiled for 5 min.

Cultures of *E. coli* transformants containing plasmid clones were grown to an optical density at 600 nm of 1.0 and then induced with 1 mM IPTG for 1 to 3 h at 37°C. Proteins were precipitated from the cultures with 20% trichloroacetic acid.

SDS-PAGE and Western blotting. Proteins were analyzed by discontinuous SDS-PAGE on 10% acrylamide gels, as described by Laemmli (15). Either gels were stained with Coomassie blue-silver stain (9) or the proteins were electrophoretically transferred onto nitrocellulose membranes for immunoblot analysis as described by Towbin et al. (37). After transfer, blots were blocked for 30 min at room temperature with 5% skim milk in TBST. The blots were washed and incubated overnight with primary antibody diluted in 1% BSA–TBST–0.05% sodium azide. Blots were incubated with horseradish peroxidase-tagged secondary antibody and developed with 4-chloronaphthol as described above.

DNA manipulations. Methods for manipulation of cloned DNA, preparation of plasmids and phage, agarose gel electrophoresis, and *E. coli* transformations and transfections were as described by Sambrook et al. (30). Restriction enzymes, T4 DNA polymerase, and T4 DNA ligase were obtained from Boehringer Mannheim Biochemicals and used according to the manufacturer's recommendations. The λ-Dash *Eco*RI fragment containing the gene encoding the 48-kDa OMP was ligated into *Eco*RI-cut λgt11 and packaged in vitro by using the Protoclone λgt11 system (Promega Corporation, Madison, Wis.). For plasmid subcloning, restriction fragments were ligated into pUC vectors (40).

DNA sequencing. DNA sequencing was performed on clones in both M13 and pUC vectors by the method of Sanger et al. (31) with the Sequenase 2.0 kit from United States Biochemicals (Cleveland, Ohio). The sequencing primers used included the universal −40 primer for M13 and pUC sequencing, as well as three oligonucleotide primers designed from previously obtained sequence data. The internal primers, 5′-TGTTGATCCCGTAGGCGACC-3′, 5′-GGTTCCCGCAT TGGACGC-3′, and 5′-GGCGATACCGATTCTGC-3′, were purchased from Research Genetics (Huntsville, Ala.). Both strands of the *aopA* gene were sequenced in their entireties.

Analysis of nucleotide and deduced amino acid sequences was performed by using the Genetics Computer Group DNA analysis programs (4).

Amino-terminal sequencing of proteins. OMs from *A. pleuropneumoniae* 5 and from the *E. coli* clone harboring pWC3 (see Fig. 4) were separated by SDS–10% PAGE. After electrophoresis, the gel was equilibrated in transfer buffer (10 mM 3-cyclohexylamino-1-propane sulfonic acid, 10% methanol, pH 11.0) for 5 min. Proteins were electroblotted onto polyvinylidene difluoride membranes (Immobilon; Millipore Corporation, Bedford, Mass.) as described by Matsudaira (17). The membrane was washed with deionized water, stained with 0.1% Coomassie blue in 50% methanol for 5 min, and then destained in 50% methanol–10% acetic acid (aldehyde free; Baker Chemical Company) for 5 to 10 min. The membrane was rinsed with deionized water and air dried, and the band containing the 48-kDa OMP was excised. Amino-terminal sequencing was performed directly from the membrane-bound protein by the Macromolecular Structure, Sequencing, and Synthesis Facility, Department of Biochemistry, Michigan State University.

Southern blots. Genomic DNAs were prepared from *A. pleuropneumoniae* type strains and from related gram-negative bacteria by standard procedures (30). DNA was digested to completion with the restriction enzyme *Eco*RI and separated on 0.7% agarose gels. DNA fragments were transferred to nitrocellu-

lose by the method of Southern (33). The insert from pYN3-6 (see Fig. 4) was labelled with digoxigenin by using the Genius DNA labelling system (Boehringer Mannheim). Filters were hybridized in 50% formamide in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 42°C and washed in 0.1% SSC–0.1% SDS at 68°C (high stringency) or were hybridized in 5× SSC without formamide at 42°C and washed in 5× SSC–0.1% SDS at 55°C (low stringency). Hybridized bands were detected with alkaline phosphatase-tagged antidigoxigenin (Boehringer Mannheim).

Nucleotide sequence accession number. The sequence data for the *A. pleuropneumoniae aopA* gene have been submitted to GenBank and assigned the accession number U24492.

RESULTS

Identification of a 48-kDa OMP common to all serotypes of *A. pleuropneumoniae*. MAbs were produced against *A. pleuropneumoniae* 5 OMs prepared by isopycnic sucrose density gradient centrifugation. Two of these MAbs, 3EH7 and 3GF2, reacted on immunoblots with an *A. pleuropneumoniae* 5 OMP antigen with an apparent molecular mass of 48 kDa. This protein is a minor component of the OM, not one of the major OMPs. Immunoblot analysis showed that this 48-kDa OMP, designated AopA for *Actinobacillus* outer membrane protein, is present in the OMs of the type strains of all 12 serotypes of *A. pleuropneumoniae* (Fig. 1). In contrast, this protein could not be detected in the OMs of *A. suis*, *E. coli*, *H. parasuis*, *Pasteurella haemolytica*, or *P. multocida* field isolates from infected swine, even when the immunoblots were developed with increased concentrations of MAb and for extended color development times (Fig. 2).

Immunogenicity of AopA in swine. OMs from *A. pleuropneumoniae* 5 were analyzed by immunoblots with sera from pigs infected with *A. pleuropneumoniae* 1A and 5 as well as sera from pigs vaccinated with several different vaccines against *A. pleuropneumoniae*. Convalescent-phase sera from both serotype 1A- and 5-infected pigs reacted strongly with the AopA antigen, as did sera from pigs immunized with a serotype 5 OM vaccine which had provided good protection against challenge with either serotype 1A or 5 (Fig. 3). Sera from pigs vaccinated with *A. pleuropneumoniae* 1A or *A. pleuropneumoniae* 5 bacterin produced in our laboratory reacted only weakly with AopA, while sera from pigs vaccinated with a commercial *A. pleuropneumoniae* bacterin showed no reaction (Fig. 3). Convalescent-phase sera from pigs infected with *A. suis*, *H. parasuis*, *E. coli*, or *P. multocida* also failed to react with the *A. pleuropneumoniae* 5 AopA protein on Western blots (data not shown).

Cloning of the gene for the 48-kDa protein. The *A. pleuropneumoniae* 5 λ-Dash genomic library was screened with a pool of five MAbs made against *A. pleuropneumoniae* 5 OMs. Of the ~5,000 plaques that were screened, nine were immunopositive. Of these nine positive clones, five produced the AopA protein, as detected in Western blots of phage plate lysates developed with MAb 3EH7.

The five positive λ-Dash clones had inserts ranging in size from 15 to 20 kb. All five clones had a common 6.0-kb fragment when digested with the restriction enzyme *Eco*RI. The 6.0-kb *Eco*RI fragment from one of the clones, λwc18, was subcloned into the *Eco*RI site of λgt11 (Fig. 4). The resulting clone, λwgt24, produced the AopA protein with IPTG induction. Restriction analysis revealed additional enzyme sites that were used to subclone smaller DNA fragments into pUC vectors (Fig. 4). Immunoblot analysis of whole-cell lysates showed that a protein of ~48 kDa was encoded by a 1.4-kb *Eco*RI-*Ava*I fragment in clone pWC3 (Fig. 4) and that this protein was expressed from the *lac* promoter of pUC18 under IPTG induction (data not shown). A clone harboring the same DNA fragment in pUC19 (pWC4 [Fig. 4]) did not produce the AopA protein even with IPTG induction. Several subclones of pWC3
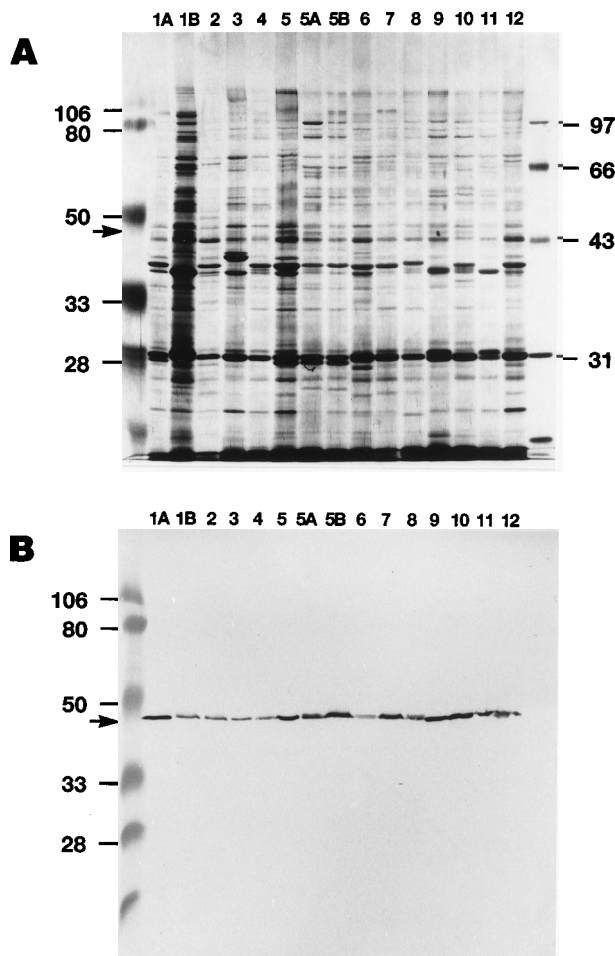

FIG. 1. AopA is produced by all serotypes of *A. pleuropneumoniae*. A Coomassie blue-silver stained gel (A) and an immunoblot developed with MAb 3EH7 (B) of OMs prepared from *A. pleuropneumoniae* serotypes 1 through 12, separated by SDS-PAGE, are shown. Serotypes are marked in each lane. Molecular mass markers are in the first (prestained markers) and last lanes, with their apparent molecular masses indicated in kilodaltons. The arrow indicates the location of the 48-kDa antigen.

produced truncated proteins detectable by Coomassie blue staining under IPTG induction (Fig. 4). However, none of these truncated proteins reacted with MAb 3EH7 on immunoblots, indicating that at least part of the epitope recognized by this antibody resides in the carboxy-terminal one-third of the AopA protein.

Sequence analysis of the *aopA* gene. The entire 1.4-kb insert from pWC3 was subcloned into M13mp18 and M13mp19 vectors for single-stranded DNA sequencing. In addition, smaller restriction fragments were subcloned into pUC18 or pUC19 and sequenced directly by using a modified method for sequencing double-stranded DNA. The sequencing strategy is outlined in Fig. 4.

When the entire DNA insert in pWC3 had been sequenced on both strands, it became apparent that this clone, which contained no in-frame stop codons, was slightly truncated. The 3′ end of the gene was sequenced in a clone containing the entire 6.0-kb *Eco*RI fragment, and the *Bsp*MI site identified downstream of the stop codon was used to clone the full *aopA* gene into pUC18 (pYN3-6 [Fig. 4]).

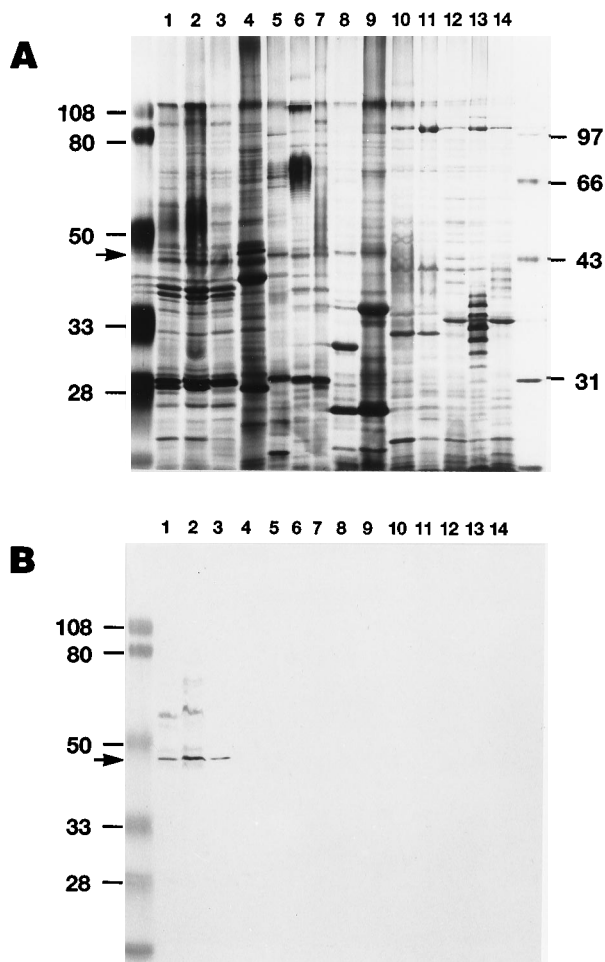The complete nucleotide sequence and the deduced amino

FIG. 2. AopA is not produced by other gram-negative swine pathogens. A Coomassie blue-silver stained gel (A) and an immunoblot developed with MAb 3EH7 (B) of OMs prepared from field isolates of gram-negative swine pathogens, separated by SDS-PAGE, are shown. Lanes: 1, *A. pleuropneumoniae* 1A; 2, *A. pleuropneumoniae* 5; 3, *A. pleuropneumoniae* 7; 4, *H. parasuis*; 5, *P. haemolytica*; 6 and 7, *A. suis*; 8 and 9, *P. multocida*; 10 to 14, *E. coli*. Molecular mass markers are in the first (prestained markers) and last lanes, with their apparent molecular masses indicated in kilodaltons. The arrow indicates the location of the 48-kDa antigen. Note that the immunoblot was overdeveloped in an attempt to detect reactive proteins in the non-*A. pleuropneumoniae* OM preparations, leading to some nonspecific background in the *A. pleuropneumoniae* lanes.

acid sequence of the *aopA* gene are shown in Fig. 5. The sequence data revealed one open reading frame of 1,347 bp, coding for a protein of 449 amino acids with a predicted molecular weight of 48,603. There is no consensus Shine-Dalgarno sequence upstream of the methionine start codon, although the AAGTAG sequence at bp 80 to 85 may be a weak Shine-Dalgarno region, and there is no characteristic promoter matching the *E. coli* consensus. Amino acid sequencing of the amino termini of both the native AopA protein from *A. pleuropneumoniae* 5 and the recombinant protein as expressed in *E. coli* under IPTG induction verified that the sequence of the AopA protein matched that predicted from the sequence of the cloned gene, with no signal sequence present (Fig. 5).

A search of the GenBank and SwissProt databases at the nucleotide and amino acid levels revealed significant homology (60% at the DNA level and 66% at the protein level) to the *nqrA* gene of *Vibrio alginolyticus*, which encodes the α subunit of a sodium-translocating NADH-ubiquinone oxidoreductase

(1). In addition, a search of the Institute for Genomic Research (Gaithersburg, Md.) *Haemophilus influenzae* genomic sequence database (6) revealed an open reading frame, designated HI0164, encoding a putative protein of unknown function that showed 76% homology at the amino acid level to *A. pleuropneumoniae* AopA and 62% homology to the *V. alginolyticus* NqrA protein. Whether the AopA protein is a part of an oxidoreductase complex in *A. pleuropneumoniae* remains to be determined.

Southern blot analysis. Chromosomal DNAs were prepared from the *A. pleuropneumoniae* type strains listed in Table 1, as well as from related gram-negative species. The genomic DNAs were digested with the restriction endonuclease *Eco*RI and separated on an agarose gel. Southern blot analysis with the *aopA* gene as a probe under high-stringency conditions showed that a single *Eco*RI fragment of 6.0 kb reacted with the probe in all *A. pleuropneumoniae* strains tested (Fig. 6). In addition, both strains of *A. suis* examined contained an ~4.2-kb *Eco*RI fragment that reacted with the *aopA* probe under high-stringency conditions, and both *P. multocida* strains contained a similarly reactive ~10-kb *Eco*RI fragment (Fig. 6). In contrast, genomic DNAs from the *E. coli* and *H. parasuis* strains tested did not react with the probe under either high (Fig. 6)- or low (data not shown)-stringency conditions.

Localization and characterization of the AopA protein. Cell fractionation studies were conducted to determine the cellular locations of both the native AopA protein in *A. pleuropneumoniae* and the recombinant protein in *E. coli*. Both the native and the recombinant proteins were located primarily in the OM fraction, as prepared by sucrose density centrifugation to separate OMs and cytoplasmic membranes (Fig. 7). In *E. coli*, there was also AopA protein, as well as degraded protein, detectable in the periplasmic fraction, probably because of the large amount of recombinant protein produced under IPTG induction (Fig. 7B). Treatment of the OMs (Fig. 7C) or a total
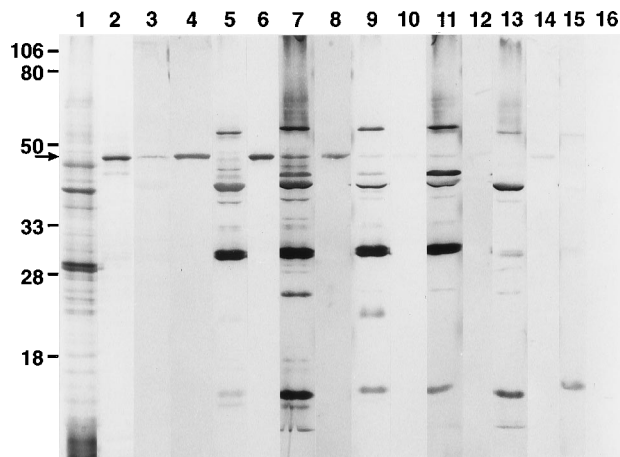
FIG. 3. AopA is immunogenic in infected swine. Coomassie blue-silver stains (lanes 1 and 2) and immunoblots (lanes 3 to 16) of OMs of *A. pleuropneumoniae* 5 (odd-numbered lanes) and of recombinant AopA protein (even-numbered lanes) are shown. Immunoblots were developed with MAb 3EH7 (lanes 3 and 4), convalescent-phase serum from a pig infected with *A. pleuropneumoniae* 1A (lanes 5 and 6), convalescent-phase serum from a pig infected with *A. pleuropneumoniae* 5 (lanes 7 and 8), serum from a pig vaccinated with serotype 5 OMs (lanes 9 and 10), serum from a pig vaccinated with a serotype 1A bacterin (lanes 11 and 12), serum from a pig vaccinated with a serotype 5 bacterin (lanes 13 and 14), and serum from a pig vaccinated with a commercial *A. pleuropneumoniae* bacterin (lanes 15 and 16). The positions of prestained molecular mass markers are indicated on the left in kilodaltons. The arrow indicates the position of the 48-kDa antigen.

Clone   **Vector**   **Protein**

Clone	Vector	kDa	MAb
λwc18	λ-dash	48	+
λwgt24	λgt11	48	+
pWC3	pUC18	48	+
pWC4	pUC19	-	-
pWC13	pUC18	34	-
pWC7	pUC18	21	-
pWC12	pUC18	-	-
pYN3-6	pUC18	48	+

FIG. 4. Physical map of major phage and plasmid clones containing the _aopA_ gene. The lines represent the inserts in the vectors listed. Restriction enzyme sites: A, _Ava_I; B, _Bam_HI; Bs, _Bsp_MI; D, _Dde_I; E, _Eco_RI; K, _Kpn_I; and M, _Mlu_I. The arrow indicate the direction of transcription from the _lac_ promoter in λgt11 and pUC clones. The apparent molecular mass of the protein produced by each clone, if any, and whether the protein reacts with MAb 3EH7 are indicated on the 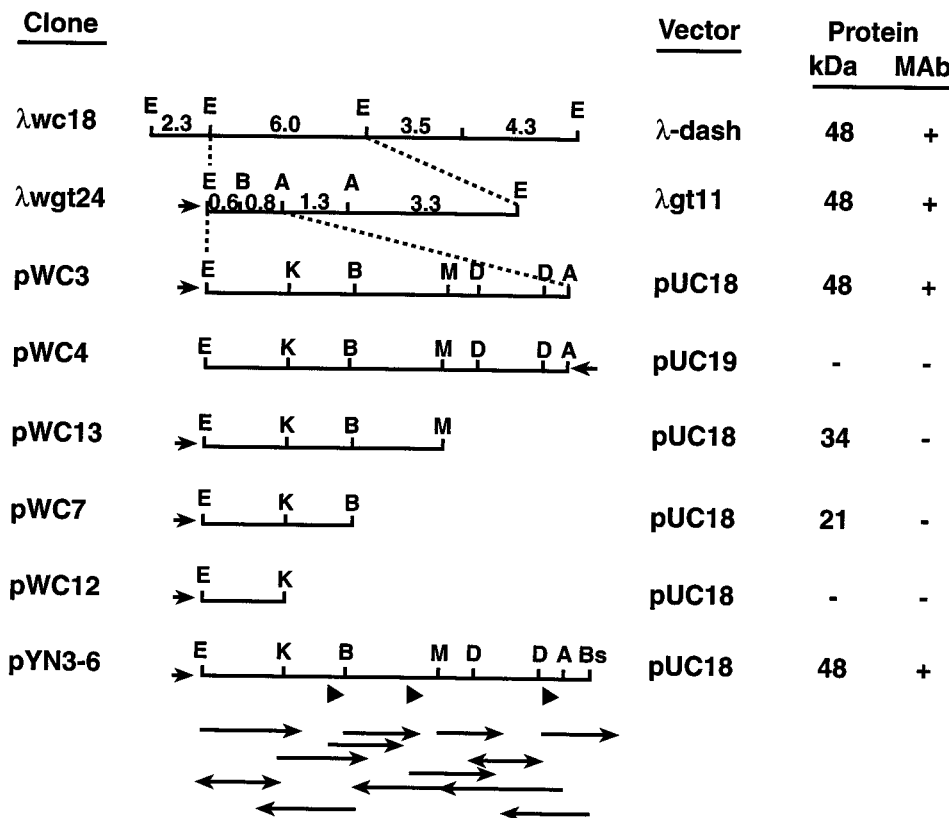right. At the bottom is an outline of the strategy for sequencing the M13 or plasmid clones by using the M13 universal −40 primer or specific oligonucleotides (triangles) designed from previously obtained sequence data.

membrane fraction containing both OMs and cytoplasmic membranes (data not shown) with 1% sarcosyl solubilized the AopA protein. Heating of _A. pleuropneumoniae_ 1A and _A. pleuropneumoniae_ 5 OMs in SDS-PAGE sample buffer at 37, 50, or 100°C for 10 min prior to separation by SDS-PAGE demonstrated that the AopA protein was not heat modifiable (data not shown).

DISCUSSION

In the present report, we describe the identification and characterization of a 48-kDa OM antigen (AopA) of _A. pleuropneumoniae_ and the cloning and expression of the respective gene (_aopA_) in _E. coli_. The AopA protein is common to all serotypes of _A. pleuropneumoniae_ but is not detected by MAbs specific for AopA in related species of gram-negative bacteria. It is immunogenic in swine infected with either _A. pleuropneumoniae_ 5 or 1A, as well as in swine vaccinated with a serotype 5 OM vaccine that provided protection against challenge with both serotypes 1A and 5 (20). In contrast, bacterin vaccines that provided at best only serotype-specific protection failed to elicit a strong antibody response against this protein.

The gene encoding the AopA protein, designated _aopA_, has been cloned in _E. coli_, and its complete nucleotide sequence has been determined. The sequence data, which extend 94 bp upstream of the start codon, show no good consensus Shine-Dalgarno, Pribnow box (−10), or −35 promoter elements. Except in the original clones in λ-Dash, expression of this protein in _E. coli_ was dependent on the _lac_ promoter in the vector and responded to IPTG induction. Since little is yet known about promoter structure in _A. pleuropneumoniae_, we cannot analyze the sequence for a consensus _A. pleuropneumoniae_ promoter. However, the data indicate that there is no promoter recognized by _E. coli_ in the cloned DNA sequence and therefore that the native promoter either differs significantly from _E. coli_ promoters or is further upstream and not contained in the clones in pUC18/19 or λgt11.

The AopA protein is localized to the OM in _A. pleuropneumoniae_ and in _E. coli_ when expressed from recombinant plasmids. It is a minor component of the OM, is not heat modifiable, and is soluble in 1% sarcosyl. It is therefore not identical to any of the _A. pleuropneumoniae_ OMPs identified in previous studies which have been conducted with OMs prepared by sarcosyl or Triton X-100 extraction of total cell membranes (3, 16, 27, 29). It is interesting that two other OMPs of _A. pleuropneumoniae_ OmlA (7) and TfbA (8), cloned by screening of _A. pleuropneumoniae_ genomic expression libraries with convalescent-phase pig sera, are also present in OMs prepared by sucrose density gradient centrifugation but are absent from OMs prepared by sarcosyl extraction procedures. This suggests that previous studies on OM antigens of _A. pleuropneumoniae_ may have failed to identify important _A. pleuropneumoniae_ antigens because these were missing from the OM preparations used (16, 28).

While AopA localizes to the OM, there is no signal sequence in this protein and thus no obvious targeting mechanism. Se-

```
                                                                                          EcoRI   .
      1                                                                                   GAATTCGTTTATTTC
                    .                    .                   .                  .                  .
     16    TATCTAAGTTACGGCTCAAGAGAAGCGATTGCGATTTTCGCAATCATATTTTTAACCCAACAAAAGTAGTGCAAATAAT

     95    ATG ATT ACA ATC AAG AAA GGC TTG GAT TTA CCT ATC GCA GGT ACA CCG GCA CAA GTA ATC
      1     M   I   T   I   K   K   G   L   D   L   P   I   A   G   T   P   A   Q   V   I
                    .                    .                   .                  .                  .
    155    CAT AAC GGC AAT ACC GTT AAT GAA GTT GCG ATG CTT GGC GAA GAA TAT GTG GGT ATG CGT
     21     H   N   G   N   T   V   N   E   V   A   M   L   G   E   E   Y   V   G   M   R
                    .                    .                   .                  .                  .
    215    CCT TCA ATG AAA GTT CGT GAA GGC GAT GTA GTG AAA AAA GGT CAG GTT CTT TTT GAA GAT
     41     P   S   M   K   V   R   E   G   D   V   V   K   K   G   Q   V   L   F   E   D
                    .                    .                 KpnI                 .                  .
    275    AAA AAG AAT CCG GGC GTT GTA TTT ACT GCT CCT GCA AGC GGT ACC GTG GTT ACT ATT AAC
     61     K   K   N   P   G   V   V   F   T   A   P   A   S   G   T   V   V   T   I   N
                    .                    .                   .                  .                  .
    335    CGC GGT GAA AAG CGT GTT CTT CAG TCG GTC GTG ATT AAA GTT GAG GGT GAT GAG CAA ATT
     81     R   G   E   K   R   V   L   Q   S   V   V   I   K   V   E   G   D   E   Q   I
                    .                    .                   .                  .                  .
    395    ACC TTT ACT CGC TAT GAA CGT GCG CAA TTA GCG TCG CTA TCA GCC GAG CAA GTG AAA CAA
    101     T   F   T   R   Y   E   R   A   Q   L   A   S   L   S   A   E   Q   V   K   Q
                    .                    .                   .                  .                  .
    455    AAT CTT ATC GAA TCG GGT TTA TGG ACT GCA TTC CGT ACT CGT CCG TTC AGT AAG GTT CCC
    121     N   L   I   E   S   G   L   W   T   A   F   R   T   R   P   F   S   K   V   P
                    .                    .                   .                  .                  .
    515    GCA TTG GAC GCG ATT CCG TCA TCA ATC TTT GTC AAT GCA ATG GAT ACC AAT CCG TTA GCG
    141     A   L   D   A   I   P   S   S   I   F   V   N   A   M   D   T   N   P   L   A
          BamHI
    575    GCG GAT CCG GAA GTG GTT TTA AAA GAG TAC GAA ACC GAT TTT AAA GAC GGT TTA ACT GTT
    161     A   D   P   E   V   V   L   K   E   Y   E   T   D   F   K   D   G   L   T   V
                    .                    .                   .                  .                  .
    635    TTA ACC CGT TTA TTT AAC GGT CAA AAA CCG GTT TAC TTA TGT AAA GAC GCG GAC AGC AAT
    181     L   T   R   L   F   N   G   Q   K   P   V   Y   L   C   K   D   A   D   S   N
                    .                    .                   .                  .                  .
    695    ATC CCG TTA AGT CCG GCG ATT GAA GGT ATC ACA ATT AAG TCG TTT AGC GGT GTT CAT CCT
    201     I   P   L   S   P   A   I   E   G   I   T   I   K   S   F   S   G   V   H   P
                    .                    .                   .                  .                  .
    755    GCC GGT TTA GTC GGT ACG CAT ATC CAC TTT GTT GAT CCC GTA GGG GCG ACC AAA CAA GTT
    221     A   G   L   V   G   T   H   I   H   F   V   D   P   V   G   A   T   K   Q   V
                    .                    .                   .                  .                  .
    815    TGG CAC TTA AAT TAT CAA GAT GTG ATT GCT ATC GGT AAA TTA TTT ACT ACA GGC GAA CTC
    241     W   H   L   N   Y   Q   D   V   I   A   I   G   K   L   F   T   T   G   E   L
                    .                    .                   .                  .                  .
    875    TTT ACC GAC CGT ATT ATT TCG CTT GCC GGT CCG CAA GTG AAA AAT CCT CGT TTA GTA CGT
    261     F   T   D   R   I   I   S   L   A   G   P   Q   V   K   N   P   R   L   V   R
                    .                    .                   .                  .                  .
    935    ACG CGT CTT GGT GCG AAT CTT TCC CAA TTA ACC GCA AAT GAG TTA AAT GCC GGT GAA AAC
    281     T   R   L   G   A   N   L   S   Q   L   T   A   N   E   L   N   A   G   E   N
                    .                    .                   .                  .                  .
    995    CGT GTG ATT TCA GGT TCG GTA TTG AGC GGT GCG ACT GCG GCG GGT CCG GTT GAT TAC TTA
    301     R   V   I   S   G   S   V   L   S   G   A   T   A   A   G   P   V   D   Y   L
                    .                    .                   .                  .                  .
   1055    GGC CGT TAC GCA TTA CAA GTA TCC GTA CTG GCG GAA GGT CGT GAG AAA GAG TTA TTC GGT
    321     G   R   Y   A   L   Q   V   S   V   L   A   E   G   R   E   K   E   L   F   G
                    .                    .                   .                  .                  .
   1115    TGG ATT ATG CCG GGT TCG GAT AAA TTC TCC ATT ACC CGT ACC GTG TTA GGC CAC TTC GGT
    341     W   I   M   P   G   S   D   K   F   S   I   T   R   T   V   L   G   H   F   G
                    .                    .                   .                  .                  .
   1175    AAA AAA TTA TTT AAC TTC ACT ACG GCA GTT CAC GGC GGT GAA CGT GCA ATG GTG CCA ATC
    361     K   K   L   F   N   F   T   T   A   V   H   G   G   E   R   A   M   V   P   I
                    .                    .                   .                  .                  .
   1235    GGT GCG TAT GAG CGT GTA ATG CCG TTA GAT ATT ATT CCT ACG TTA TTA CTT CGT GAT TTA
    381     G   A   Y   E   R   V   M   P   L   D   I   I   P   T   L   L   L   R   D   L
                    .                    .                   .                  .                  .
   1295    GCT GCC GGC GAT ACC GAT TCT GCA CAA AAC TTA GGT TGT TTA GAG CTG GAT GAA GAA GAT
    401     A   A   G   D   T   D   S   A   Q   N   L   G   C   L   E   L   D   E   E   D
                    .                    .             AvaI                     .                  .
   1355    TTG GCA TTA TGT ACT TAT GTT TGC CCG GGT AAA AAT AAC TAC GGT CCG ATG TTA CGT GCT
    421     L   A   L   C   T   Y   V   C   P   G   K   N   N   Y   G   P   M   L   R   A
                    .                    .                   .                  .                  .
   1415    GCG TTA GAG AAG ATC GAG AAG GAA GGT TAA GAAAATGGGTTTAAAAAAATCTTTTTGAAAAGATGGAACC
    441     A   L   E   K   I   E   K   E   G   *
          BspMI
   1484    TGCGTTTCAGAAAGGTGGAAAATATGAGAAAT
```

FIG. 5. Nucleotide sequence and predicted amino acid sequence of the *aopA* gene. The amino acids that were confirmed by amino-terminal sequencing of the native AopA protein from *A. pleuropneumoniae* 5 and the recombinant protein are italicized and underlined. A potential transcription termination stem-loop structure is double underlined.
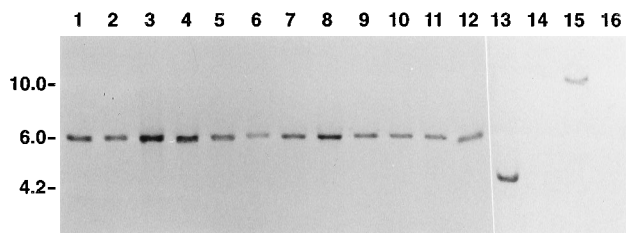
FIG. 6. The *aopA* gene is found in all serotypes of *A. pleuropneumoniae*, as well as in *A. suis* and *P. multocida*. A Southern blot of genomic DNAs from all 12 serotypes of *A. pleuropneumoniae* as well as related gram-negative bacteria, digested with the restriction enzyme *Eco*RI and probed with the *aopA* gene from *A. pleuropneumoniae* 5 under high-stringency conditions, is shown. Lanes 1 to 12, *A. pleuropneumoniae* serotype type strains; lane 13, *A. suis*; lane 14, *H. parasuis*; lane 15, *P. multocida*; lane 16, *E. coli* K99. Lanes 1 to 12 contain 2.5 μg of DNA per lane; lanes 13 to 16 contain 10 μg of DNA per lane.

cretion of proteins in gram-negative bacteria generally occurs by one of three distinct systems. The general secretion pathway system secretes proteins in a two-step process, utilizing the general export pathway, or Sec pathway, to export proteins across the cytoplasmic membrane and then using up to 14 different gene products to secrete proteins through the OM (26). Proteins secreted by this pathway have a classic signal sequence and are posttranslationally processed by signal peptidases (26). Two examples of this type of secretion in *A. pleuropneumoniae* are the ROB-1 β-lactamase (2) and the OmlA lipoprotein (7). The signal-peptide-independent pathway secretes proteins through both the cytoplasmic membrane and OM simultaneously, utilizing several accessory proteins to form a secretion "pore" (41). Proteins secreted by this pathway

have no classical N-terminal signal sequence; rather, the secretion signal appears to be located in the C-terminal portion of the protein (41). The Apx cytotoxins of *A. pleuropneumoniae* are examples of this type of secretion mechanism (6a). A third secretion pathway has been described for virulence antigens of *Yersinia*, *Shigella*, and *Salmonella* spp. (1a). Proteins secreted by this pathway do not require a signal sequence and generally either are completely secreted into the growth medium or remain loosely associated with the outer membrane (1a). It is possible that the *A. pleuropneumoniae* AopA protein is secreted by an analogous system, although there are as yet no data demonstrating that *A. pleuropneumoniae* contains genes homologous to those required for this secretion pathway.

Immunoblots with MAbs specific for the AopA protein showed no antigenically related protein in OMs from other species in the *Haemophilus-Actinobacillus-Pasteurella* family of bacterial pathogens. However, Southern blot analysis detected DNA homologous to *aopA* in *A. suis* and *P. multocida*. These results suggest that *A. suis* and *P. multocida* may produce proteins homologous to, but antigenically distinct from, AopA. This should not preclude the potential use of the AopA protein, possibly in combination with specific MAbs, in a serodiagnostic test for *A. pleuropneumoniae*.

Several criteria can logically be employed for the identification of antigens which have the potential to elicit a cross-protective immune response against *A. pleuropneumoniae*. First, the antigen should be present in all strains of *A. pleuropneumoniae*, regardless of serotype. Second, it should be immunogenic in infected swine, indicating that it is indeed expressed during infection. Third, it should be surface exposed and therefore a potential target for either serum bactericidal activity or opsonophagocytosis. By these criteria, the AopA antigen is a good candidate for further study as a purified protein vaccine against *A. pleuropneumoniae*.
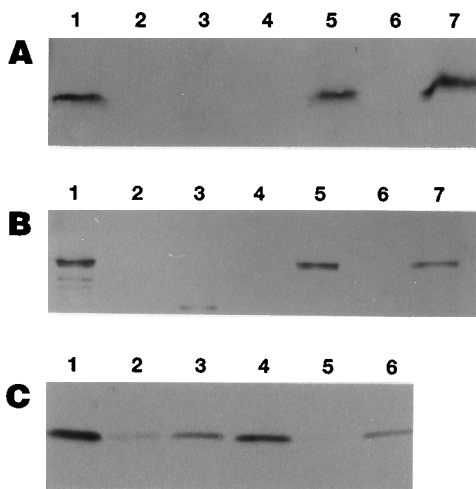
FIG. 7. AopA is found in the OM in *A. pleuropneumoniae* 5 and in *E. coli* containing the cloned *aopA* gene and is soluble in 1% sarcosyl. (A and B) Immunoblots of *A. pleuropneumoniae* 5 (A) and *E. coli* JM103/pYN3-6 (B) cell fractions developed with MAb 3EH7. Lanes: 1, whole cell; 2, cell-free culture supernatant; 3, periplasm; 4, cytoplasm; 5, total cell membranes; 6, cytoplasmic membrane; 7, OM. In panel A, lane 1 contains the equivalent of 60 μl of bacterial culture or ~5 × 10^7 CFU, and lanes 2 to 7 contain the equivalent of 240 μl of culture. In panel B, lane 1 contains the equivalent of 15 μl of bacterial culture, or ~2 × 10^7 CFU, and lanes 2 to 7 contain the equivalent of 60 μl of culture. (C) Immunoblot developed with MAb 3EH7. Lanes: 1, *A. pleuropneumoniae* 5 OM prepared by sucrose density gradient centrifugation; 2, serotype 5 OM treated with 1% sarcosyl, insoluble fraction; 3, serotype 5 OM treated with 1% sarcosyl, soluble fraction; 4 to 6, same as lanes 1 to 3, respectively, except with serotype 1A OM. Lanes 1 and 4 each contain 5 μg of OM protein; lanes 2, 3, 5, and 6 contain the cell equivalent of 5 μg of OM protein after sarcosyl treatment.

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