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Cloning, Sequencing, Expression, and Protective Capacity of the *oma87* Gene Encoding the *Pasteurella multocida* 87-Kilodalton Outer Membrane Antigen

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Membrane proteins of *Pasteurella multocida* have been shown previously to elicit protective immunity. We have identified an 87-kDa outer membrane antigen, Oma87, which is present in all 16 serotypes of *P. multocida*. The gene encoding this protein was cloned and sequenced and found to have significant similarity to the D15 protective surface antigen of *Haemophilus influenzae*. Oma87 was localized to the outer membrane of the cell, and proteinase K treatment suggested that the protein is surface exposed. Native and recombinant Oma87 were strongly immunostained by convalescent-phase antiserum, indicating that the protein is expressed in vivo. Specific Oma87 antiserum protected mice against homologous, lethal *P. multocida* challenge. These results suggest that Oma87 is a protective outer membrane antigen of *P. multocida*.

The animal pathogen *Pasteurella multocida* is associated with a wide range of diseases, including fowl cholera of poultry and wild fowl, atrophic rhinitis of swine, and hemorrhagic septicemia of cattle and buffaloes. Fowl cholera is a highly contagious disease which results in significant economic losses to the poultry industry worldwide (17). Presently, strategies for the control of this disease include the use of killed whole-cell vaccines, which afford homologous protection, and attenuated live vaccines. Although cross-protective immunity is stimulated by the empirically derived, attenuated live vaccines, reversion to virulence occurs, causing outbreaks of fowl cholera in vaccinated flocks (18).

Few *Pasteurella* antigens or virulence factors which are important for pathogenesis or immunity in pasteurellosis have been identified. Capsular type D strains produce a dermonecrotic toxin which plays a crucial role in the pathogenesis of atrophic rhinitis in pigs (35). The production of neuraminidase by some *P. multocida* strains may also contribute to the disease process (19), while the importance of capsules and lipopolysaccharide (LPS) remains controversial. Antigens associated with the outer membrane (OM) fractions of in vivo-grown strains elicit cross-protective immunity, whereas in vitro-grown strains afford only homologous protection, suggesting that OM antigens expressed in vivo are important in immunity against pasteurellosis (16, 30, 31). OM proteins (OMPs) are thought to be the major antigens involved in inducing a cross-protective response, since immunization with LPS alone induced only partial protection (29). Few OMPs of *P. multocida* have been characterized. A 37-kDa OMP is thought to be the major porin (7, 26), a 50-kDa OMP was reported to have antiphagocytic activity (37), and Lu et al. (24) demonstrated that a monoclonal antibody against a 37.5-kDa OMP was protective in animal models. OM antigens expressed under iron-limiting conditions have been identified and appear to play a role in immunity against pasteurellosis (8, 32). Recently, a 16-kDa OMP which shared a high degree of similarity with the P6 protective OMP of *Haemophilus influenzae* has been characterized and is thought to be immunogenic (21).

Since it has been demonstrated that *P. multocida* OM fractions are able to induce an immune response, our studies have concentrated on identifying the individual antigens involved in eliciting such a response. In this paper, we report the cloning and sequencing of a gene encoding an 87-kDa OM antigen (Oma87) of *P. multocida* and its expression in *Escherichia coli*. Furthermore, we present evidence that this protein may be a target for protective immunity.

MATERIALS AND METHODS

Bacterial strains and plasmids. *P. multocida* serotype A:1 strain PBA100 (20) and serotype A:3 strain PBA101 were isolated from Australian cases of fowl cholera and cultured routinely at 37°C in nutrient broth no. 2 or nutrient agar (Oxoid) with 0.3% added yeast extract. *P. multocida* strains were maintained on horse blood agar plates and stored at 4°C. *E. coli* DH5α (Bethesda Research Laboratories, Gaithersburg, Md.) strains were cultured at 37°C in Luria broth (LB) or on LB agar (1.2%, wt/vol). *E. coli* strains harboring plasmids were cultured with the addition of 100 μg of ampicillin per ml. Plasmids pUC18 and pUC19 (38) were used as standard cloning vectors.

Recombinant DNA techniques. Chromosomal DNA was isolated from *P. multocida* as described previously (2). Plasmid DNA from *E. coli* strains was isolated by the alkaline lysis method (5). Basic molecular cloning techniques were carried out by standard protocols (2). *E. coli* strains were transformed with plasmid DNA following electroporation with a Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, Calif.) set at 200 Ω, 1.80 kV, and 25 μF by the method described previously (12).

Construction and screening of PBA100 library. Genomic DNA from *P. multocida* PBA100 was partially digested with *Sau*3A (Boehringer GmbH, Mannheim, Germany). DNA fragments ranging from 2.0 to 9.0 kb were excised from agarose gels, purified with the Bio 101 (Bresatec, Australia) Geneclean Kit, and ligated to *Bam*HI-digested, dephosphorylated pUC18. Electrocompetent *E. coli* DH5α cells were transformed with the ligation mix, incubated for 2 h at 37°C to allow expression, and then plated onto LB agar containing ampicillin. Transformants were screened by colony immunoassay (2), with rabbit antiserum, PM100, prepared against whole-membrane fractions of PBA100.

SDS-PAGE and Western blotting (immunoblotting). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (23) with a Bio-Rad Protean II apparatus. Membrane fractions were loaded at 15 μg of protein per lane, while bacterial cultures were standardized by absorbance. SDS-polyacrylamide gels were either stained with Coomassie brilliant blue or electrophoretically transferred onto nitrocellulose (0.45 μm) in a Bio-Rad TransBlot cell for Western blotting. Transferred proteins were immunostained with specific antisera at a dilution of 1:100 and peroxidase-conjugated sheep anti-mouse immunoglobulins, peroxidase-conjugated sheep anti-rabbit immunoglobulins (Silenus Laboratories, Melbourne, Australia), or peroxidase-conjugated goat anti-chicken immunoglobulins (Sigma Chemical Co., St. Louis, Mo.) as described previously (6). Elution of antibodies from nitrocellulose fol-

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lowing Western blotting was carried out as described by Beall and Mitchell (4) with the following modifications. Prior to the elution of antibodies, strips of nitrocellulose were washed in 0.1 M Tris-HCl (pH 7.4) containing 0.5 M NaCl. Eluted antibodies were diluted 1:2 with 5% (wt/vol) skim milk in Tris-buffered saline (pH 7.4) containing 0.05% (vol/vol) Tween 20 and used immediately.

DNA sequencing and analysis. Sequencing was performed on purified plasmid DNA with synthetic oligonucleotides and the *Taq* DyeDeoxy Terminator Cycle kit (Applied Biosystems Inc., Foster City, Calif.). Oligonucleotides were synthesized with an Applied Biosystems model 392 DNA-RNA synthesizer. Sequence reaction products were analyzed with an Applied Biosystems model 373 automated DNA sequencer. Individual sequences were aligned and assembled with Sequencher 3.0 (Gene Codes Corp., Ann Arbor, Mich.). Comparison of the nucleotide and derived protein sequences with sequences within GenBank and EMBL databases was carried out by use of the BLAST (1) and FASTA (28) programs through the Australian National Genomic Information Service (University of Sydney, Australia).

Protein analysis. Computer analysis of the derived protein sequences was carried out through the Australian National Genomic Information Service by use of programs within the Genetics Computer Group Sequencing Analysis Software Package (GCG, Inc., Madison, Wis.). Hydropathy plots, from the algorithm of Kyte and Doolittle (22) with a window size of 19, were generated with DNA Strider 1.2 (27).

Preparation of membrane fractions. Membrane fractions were purified by stepwise sucrose gradient centrifugation. *P. multocida* and *E. coli* overnight cultures were pelleted, washed with phosphate-buffered saline (pH 7.2; PBS), and again pelleted. Cell pellets were resuspended in 6 ml of 20% sucrose in 10 mM Tris-HCl (pH 8.0) containing 40 mM DNase and incubated at 22°C for 15 min. The cells were then disrupted by two passages through a French pressure cell (Aminco, Silver Spring, Md.) at 138 MPa, and the cell debris was removed by centrifugation at 3,000 × *g* for 15 min. Three milliliters of supernatant was applied to a stepwise gradient of 25% and 70% sucrose containing 3 mM EDTA and centrifuged at 230,500 × *g* for 2 h at 4°C. The cytoplasmic fraction was collected above the 25% sucrose step and stored at −20°C. The whole-membrane fraction was collected above the 70% sucrose step, diluted in 3 mM EDTA, and pelleted at 230,500 × *g* for 2 h at 4°C. Pelleted membranes were resuspended in sterile distilled water and stored at −20°C. The protein concentration of membrane fractions was determined by the Bradford assay (Bio-Rad), with bovine serum albumin as a standard. OM fractions of PBA100 were prepared as described previously (32).

Proteinase K treatment. Proteinase K treatment of cells was performed as described by Barbour et al. (3) with the following modifications. Log-phase cultures were washed twice with PBS, treated with 0.5 M HCl for 2 h, and washed a further two times in PBS containing 5 mM $MgCl_2$ (PBS-$MgCl_2$). Cells were resuspended in PBS-$MgCl_2$, proteinase K (Sigma) was added to a final concentration of 0.4 mg/ml, and the cells were incubated at 37°C for 30 min. Phenylmethylsulfonyl fluoride, at a final concentration of 1 mg/ml, was added to inhibit the proteinase K activity. The cells were then washed twice with PBS-$MgCl_2$, resuspended in a final volume of 50 μl, and stored at −20°C.

Passive immunization. Female New Zealand White rabbits were injected intramuscularly on day 1 with OM antigens which consisted of 30 μg of membrane fraction in 0.1 ml of PBS emulsified with 0.1 ml of Freund's incomplete adjuvant. On day 14, the rabbits were bled and then immunized with 30 μg of membrane fraction in 0.2 ml of PBS without adjuvant. Sera were collected on day 40, and the presence or absence of anti-Oma87 antibodies was assessed by Western blotting. Prior to use in immunization experiments, the antisera were filter sterilized, aliquoted, and stored at either 4 or −20°C.

Female BALB/c outbred mice, 6 to 8 weeks of age, were used in passive immunization experiments. To monitor the levels of circulating antibodies, mice were given 200 μl of undiluted antiserum intraperitoneally and then bled from the orbital plexus every hour for 8 h. For passive immunization experiments, mice were injected intraperitoneally with 200 μl of undiluted antiserum and then challenged after 6 h with 100 50% infective doses of PBA100 or PBA101. Unimmunized control mice were injected with 200 μl of PBS. Mice which were moribund and deemed incapable of survival were euthanized in accordance with animal ethics requirements.

Nucleotide sequence accession number. The sequence data for the *P. multocida oma87* gene have been submitted to the GenBank database and assigned the accession number U60439.

RESULTS

Cloning and mapping of the *oma87* gene. A genomic DNA library of *P. multocida* PBA100 was constructed in pUC19 and introduced into *E. coli* DH5α. The library was screened with PM100 antiserum, previously shown to protect mice in passive immunization studies (unpublished observation), and recombinant clones which reacted strongly with this antiserum were analyzed by SDS-PAGE and Western blotting. Clone PBA1014 expressed an 87-kDa protein that was strongly immunostained by PM100. The protein was localized to the OM of *P. multocida* (see below) and thus designated Oma87, the 87-kDa OM antigen of *P. multocida*. The gene which encoded Oma87 was localized to a 2.85-kb *Hin*dIII-*Sca*I fragment of pPBA1014. This fragment was cloned into the *Hin*dIII-*Hin*cII sites of pUC18 to yield the plasmid pPBA1137. Western blot analysis with PM100 showed that *E. coli* harboring pPBA1137 (PBA1137) expressed the Oma87 protein (Fig. 1, lane 1), which was not present in the *E. coli* control strain LBA30 (Fig. 1, lane 2) but which corresponded to a band of similar size in PBA100 (Fig. 1, lane 3).

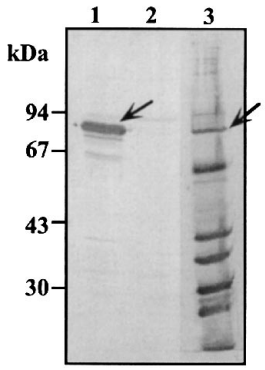

FIG. 1. Western blot analysis of whole-cell lysates immunostained with rabbit antiserum PM100. Lanes: 1, *E. coli* PBA1137 (*oma87* clone); 2, *E. coli* LBA30 (control); 3, *P. multocida* PBA100. Arrows indicate Oma87. The positions of standard molecular mass markers are shown on the left. The original was scanned with an Epson GT-8000 scanner and the Adobe Photoshop 2.5.1 LE program.

DNA sequence analysis. The nucleotide sequence of the 2.85-kb insert of pPBA1137 and the deduced amino acid sequence revealed a putative promoter region and two open reading frames (ORFs), one of which remained open to the end of the sequence obtained. The complete ORF began with an ATG initiation codon, comprised 2,372 nucleotides, and encoded a protein of 789 amino acids. This ORF was designated the *oma87* gene, encoding the Oma87 protein. In pPBA1137, the *oma87* gene was cloned in the opposite orientation with respect to the *lac* promoter, thus indicating that the sequence upstream of the *oma87* coding region contained a functional promoter(s). Putative −35 and −10 promoter sequences were identified within the upstream region of *oma87*, and a consensus ribosomal binding sequence was located 11 bp upstream of the start codon (Fig. 2). Examination of the 3′ terminus of *oma87* revealed a putative Rho-independent transcriptional terminator (Fig. 2). An intergenic region of 107 bp was located downstream of *oma87* and was followed by the start of a second ORF, of which 33 bp were present within the 2.85-kb fragment of pPBA1137.

Analysis of Oma87 amino acid sequence. From the deduced amino acid sequence, the Oma87 protein had a predicted molecular mass of 87,447 Da. Examination of the N terminus of Oma87 revealed an 18-amino-acid stretch (M-1 to A-18) which consisted of two basic lysine residues followed by a stretch of hydrophobic residues, characteristic of signal peptides. On the basis of the proposed consensus cleavage site for peptidase I (39), the most probable cleavage site lies between A-18 and A-19. The mature protein thus has a deduced molecular mass of 85,553 Da, which is in good agreement with the molecular mass of 87 kDa estimated by SDS-PAGE. Hydropathy analysis (Fig. 3) of Oma87 showed a typical hydrophobic stretch at the N terminus, which corresponded to the putative signal peptide

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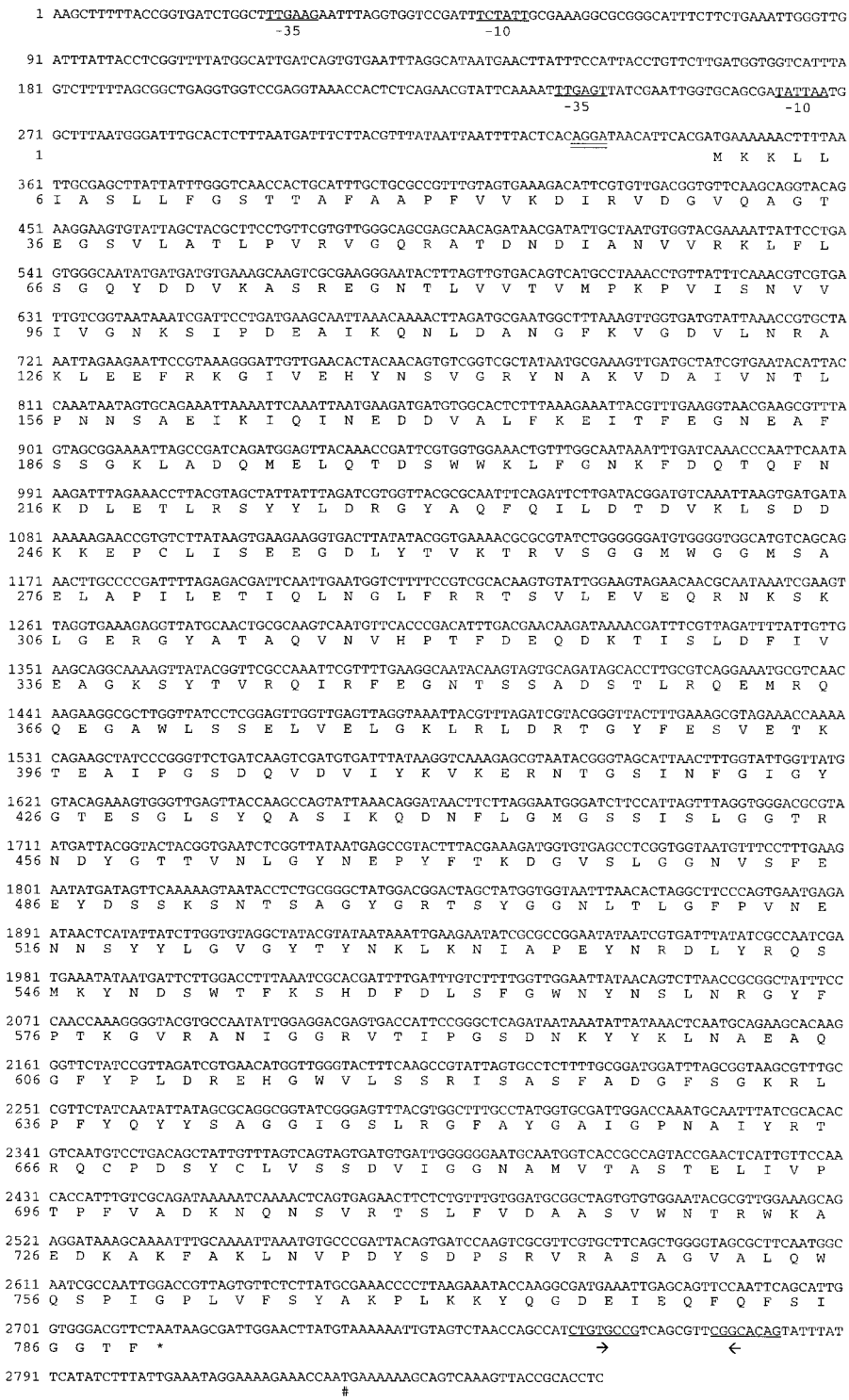

FIG. 2. Nucleotide sequence of the 2.85-kb fragment of pPBA1137 and the deduced amino acid sequence for the ORF from position 345 to 2711 comprising the *oma87* gene. Potential −35 and −10 promoter sequences are underlined. The double-underlined bases indicates the ribosome binding site, and the asterisk indicates the TAA stop codon. Arrows indicate the inverted repeats that comprise the putative Rho-independent transcriptional terminator. The numeral symbol (#) indicates the ATG start codon of the second reading frame identified as the *skp* gene of *P. multocida.*

and hydrophilic regions, with smaller intervening hydrophobic stretches throughout the protein. The hydrophilic peaks corresponded to predicted surface domains generated by the method of Emini et al. (13) (data not shown). The last 10 amino acid residues at the C terminus, Q-F-Q-F-S-I-G-G-T-F, constituted a sequence characteristic of bacterial OMPs which is important for their correct assembly into the membrane (33) (Fig. 4).

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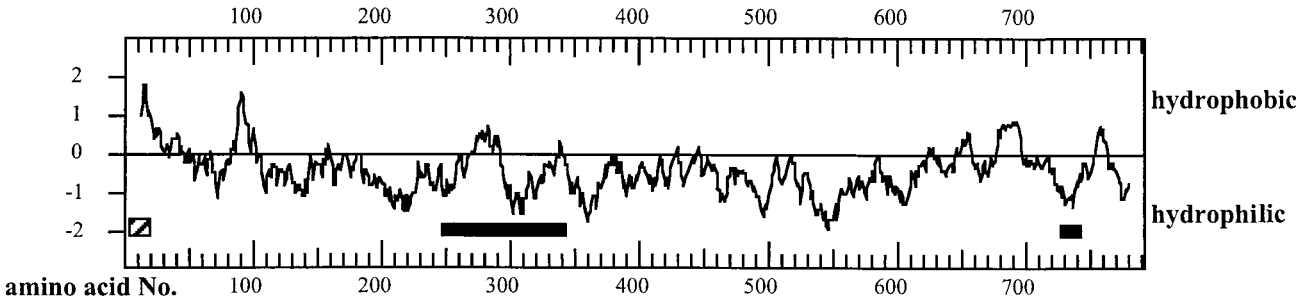

FIG. 3. Hydropathy profile of Oma87 generated by the algorithm of Kyte and Doolittle (22) with a window size of 19. The putative signal peptide is indicated by the hatched bar, and the solid bars indicate the variable domains found in Oma87 and D15.

Database searches with the nucleotide and deduced amino acid sequences of Oma87 revealed significant similarity to the sequences of the D15 protective surface antigen of *H. influenzae* type b (D15b) (14) and the D15 homolog of *H. influenzae* Rd (D15Rd) (15). The sequence of Oma87 was 75% identical to those of D15b and D15Rd. Because of the significant similarity (99.5%) between the D15b and D15Rd amino acid sequences, further comparison of Oma87 was made only with D15b. Alignment of the D15b and Oma87 proteins revealed conserved regions at the N terminus, notably the signal peptide and the putative cleavage site, while at the C terminus, D15b also possessed the conserved 10-amino-acid sequence characteristic of bacterial OMPs (Fig. 4). Variable regions between the two proteins occurred between amino acid residues 247 to 345 and 730 to 743 (Fig. 4). These variable domains corresponded to hydrophilic regions in the hydropathy profiles of Oma87 (Fig. 3) and D15b (data not shown). On the basis of additional sequence data, the second ORF found downstream of *oma87* was identified as part of the *skp-firA* operon of *P. multocida* (11).

Expression, localization, and immunological analysis of Oma87. Western blot analysis with PM100 antiserum or eluted Oma87 antibodies revealed that the recombinant *E. coli* clone PBA1137 expressed an increased amount of Oma87 compared with the wild-type strains PBA100 and PBA101 (Fig. 1 and 5). Since the *oma87* gene was cloned in the opposite orientation with respect to the *lac* promoter, the increased expression may be due to the high copy number of pPBA1137. Alternatively, the regulation of *oma87* in *E. coli* may be altered or absent, resulting in increased levels of protein.

To localize Oma87 in *P. multocida*, inner membrane (IM), OM, and cytoplasmic fractions were prepared from PBA100 and PBA101. The fractions were examined for the presence of Oma87 by Western blotting with antibodies that had been eluted from the recombinant Oma87 protein of PBA1137. The eluted antibodies immunostained predominantly an 87-kDa protein in the OM fractions of PBA100 and PBA101, which was assumed to be Oma87 (Fig. 5). In comparison, the IM fractions contained reduced amounts of Oma87 (Fig. 5). Neither the cytoplasmic fractions nor the control *E. coli* harboring pUC18 (LBA30) contained Oma87 (Fig. 5). Western blot analysis of total membrane and cytoplasmic fractions of the PBA1137 immunostained with PM100 revealed that the recombinant Oma87 was located predominantly within the membrane fractions (data not shown). Proteinase K treatment of PBA100 further supported an OM location of Oma87. Western blot analysis with rabbit antiserum against membrane fractions from the PBA1137 *E. coli* clone (CR3; see below) revealed that Oma87 was absent in whole-cell lysates of PBA100 previously treated with proteinase K but present in lysates of control cells that were mock treated with proteinase K (Fig. 6).

Western blot analysis of the 16 *P. multocida* serotypes with antibodies eluted from the recombinant Oma87 revealed that all serotypes expressed an 87-kDa protein, assumed to be homologs of Oma87 (data not shown). In addition, a convalescent-phase chicken antiserum, CA101, was able to immunostain both native and recombinant Oma87 (Fig. 7).

```
          1                                                          60
Oma87     MKKLLIASLLFG-STTAFAAPFVVKDIRVDGVQAGTEGSVLATLPVRVGQRATDNDIANV
D15       ************TT**V******A*********GDL*QQIR*S****A***V****V**I

          61                                                         120
Oma87     VRKLFLSGQYDDVKASREGNTLVVTVMPKPVISNVVIVGNKSIPDEAIKQNLDANGFKVG
D15       **S**V**RF*****HH**DV***S*VA*SI**D*K*K**SI**T**L************

          121                                                        180
Oma87     DVLNRAKLEEFRKGIVEHYNSVGRYNAKVDAIVNTLPNNSAEIKIQINEDDVALFKEITF
D15       ***I*E**N**A*SVK***A*******T*EP********R***L*******K*KLASL**

          181                                                        240
Oma87     EGNEAFSSGKLADQMELQTDSWWKLFGNKFDQTQFNKDLETLRSYYLDRGYAQFQILDTD
D15       K***SV**ST*QE*****P******W****EGA**E***QSI*D***NN***KA**TK**

          241                                                        300
Oma87     VKLSDDKKEP--CLISEEGDLYTVKTRVSGGMWGGMSAELAPILETIQLNGLFRRTSVLE
D15       *Q*N*E*TKVNVTIDVN**LQ*DLRSARII*NL*******E*L*SALH**DT***SDIAD

          301                                                        360
Oma87     VEQRNKSKLGERGYATAQVNVHPTFDEQDKTISLDFIVEAGKSYTVRQIRFEGNTSSADS
D15       **NAI*A*******GS*T**SV*D**DAN**LAITLV*D**RRL****L******V****

          361                                                        420
Oma87     TLRQEMRQQEGAWLSSELVELGKLRLDRTGYFESVETKTEAIPGS-DQVDVIYKVKERNT
D15       **********T*YN*Q******I******E**T**NRIDP*N**N*E***V********

          421                                                        480
Oma87     GSINFGIGYGTESGLSYQASIKQDNFLGMGSSISLGGTRNDYGTTVNLGYNEPYFTKDGV
D15       **************I*****V*******T*AAV*IA**K*****S*****T*********

          481                                                        540
Oma87     SLGGNVSFEEYDSSKSNTSAGYGRTSYGGNLTLGFPVNENNSYYLGVGYTYNKLKNIAPE
D15       ******F**N**N***D**SN*K**T**S*V*************V*L*H****IS*F*L*

          541                                                        600
Oma87     YNRDLYRQSMKYNDSWTFKSHDFDLSFGWNYNSLNRGYFPTKGVRANIGGRVTIPGSDNK
D15       ***N**I****FKGNG-I*TN***F******************K*SL************

          601                                                        660
Oma87     YYKLNAEAQGFYPLDREHGWVLSSRISASFADGFSGKRLPFYQYYSAGGIGSLRGFAYGA
D15       ****S*DV********D*L**V*AKA**GY*N**GN********T*T*************S

          661                                                        720
Oma87     IGPNAIYRTRQCPD-SYCLVSSDVIGGNAMVTASTELIVPTPFVADKNQNSVRTSLFVDA
D15       *******AEHGNGNGTFKKV*********IT***A*********S**S**T*********

          721                                                        780
Oma87     ASVWNTRWKAEDK--AKFAKLNVPDYSDPSRVRASAGVALQWQSPIGPLVFSYAKPLKKY
D15       ******H**SDKSGLDNNVLKSL***GKS**I***T**GF****************I***

          781        796
Oma87     QGDEIEQFQFSIGGTF
D15       EN*DV********S*
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FIG. 4. Alignment of *P. multocida* Oma87 and D15 from *H. influenzae*. Asterisks indicate identical amino acid residues, while underlined residues indicate conserved amino acid changes. Amino acid residues from 1 to 18 constitute the putative signal peptide, the cleavage site of which is predicted to be between A-18 and A-19; the arrow indicates the first amino acid residue of the mature protein. The 10 last amino acids at the C terminus, shown in bold, constitute a sequence which is thought important for correct assembly of the protein into the OM.

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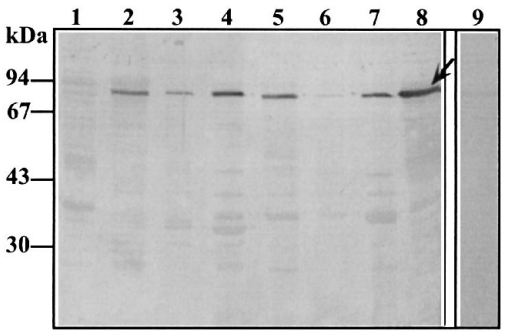

FIG. 5. Western blot analysis of whole-cell lysates (WC) and membrane fractions (IM, DM, and cytoplasmic [CF]) immunostained with antibodies eluted from recombinant Oma87 previously immunostained with PM100. Lanes: 1, LBA30 WC; 2, PBA100 WC; 3, PBA100 IM; 4, PBA100 OM; 5, PBA101 WC; 6, PBA101 IM; 7, PBA101 OM; 8, PBA1137 WC; 9, PBA100 CF. The arrow indicates Oma87. The positions of standard molecular mass markers are shown on the left. The originals were scanned with an Epson GT-8000 scanner and the Adobe Photoshop 2.5.1 LE program.

Protective capacity of Oma87. Passive immunization studies with BALB/c mice as an animal model were used to assess the protective capacity of Oma87. Antiserum from rabbits injected with membrane fractions that were prepared from PBA1137 (CR3) contained anti-Oma87 antibodies, while antiserum from rabbits injected with LBA30 membrane fractions (CR6) did not contain anti-Oma87 antibodies (Fig. 8). CR6 was therefore used as the control antiserum in passive immunization experiments. The titer of circulating antibodies was assessed by Western blot analysis after mice were immunized with undiluted antiserum. The Oma87 antibody titer was 50 at 4 h after the initial injection, and after 6 h, the titer was 100. Therefore, 6 h following the initial injection, mice were challenged with lethal doses of *P. multocida*. Mice that received CR6 antiserum were not protected against either a homologous or a heterologous lethal challenge. In contrast, mice that received CR3 were protected against a homologous challenge. However, CR3 was not able to protect the mice against a higher challenge dose with PBA100 nor against a heterologous challenge (Table 1).

DISCUSSION

In the present study, we have identified an 87-kDa OM antigen of *P. multocida*, Oma87. The *oma87* gene encoding this protein was cloned and sequenced, and the protective capacity of Oma87 was assessed. The nucleotide and the deduced amino acid sequences of the cloned *oma87* gene showed extensive similarity to those of the 80-kDa D15 protective surface antigen of *H. influenzae* (14, 15).

On the basis of its amino acid sequence and hydropathy profile (Fig. 2 and 3), we propose that Oma87 is an OM antigen. As is characteristic of proteins translocated across the IM, the first 18 amino acid residues at the N terminus of Oma87 constitute a putative signal peptide, which when cleaved would result in a mature protein of 85,553 Da. The C terminus of Oma87 is highly characteristic of OMPs. The last residue at the C terminus, phenylalanine, is highly conserved among OMPs and is essential for stability and correct assembly of the protein into the OM (10, 33). According to Struyve et al. (33), the last 10 amino acid residues of membrane proteins, including the terminal phenylalanine, have conserved hydrophobic residues which are important for incorporation of the protein in the membrane. The last 10 residues, Q-F-Q-F-S-I-G-G-T-F, of Oma87 (Fig. 4) contain the conserved hydrophobic residues at positions 2, 4, 6, and 10 in that sequence. Although the glycine at position 8 is not hydrophobic, given the overall conservation of the other residues, particularly the terminal phenylalanine, it is most probable that these residues form a membrane-spanning region, with the C terminus of Oma87 possibly directed towards the periplasm (10). The conserved C-terminal amino acids are also present in the *H. influenzae* D15 surface protein.

The hydropathy profile of Oma87 shows a number of hydrophilic domains throughout the protein (Fig. 3). Hydrophilic domains within OMPs often correspond to cell surface-exposed domains which are highly variable in their amino acid composition when compared with similar OMPs (36). A number of hydrophilic peaks of Oma87 corresponded to predicted surface-exposed domains. Furthermore, as evident from the

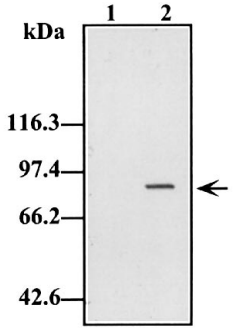

FIG. 6. Western blot analysis of PBA100 proteinase K-treated PBA100 (lane 1) and PBA100 proteinase K control (lane 2) immunostained with Oma87-specific antiserum CR3. The arrow indicates Oma87. The positions of standard molecular mass markers are shown on the left. The originals were scanned with an Epson GT-8000 scanner and the Adobe Photoshop 2.5.1 LE program.

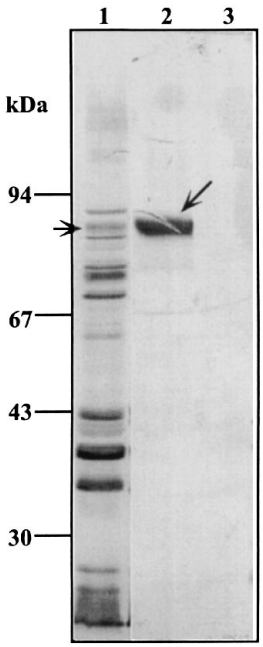

FIG. 7. Western blot analysis of whole-cell lysates immunostained with chicken convalescent-phase antiserum CA101. Lanes: 1, PBA100; 2, PBA1137; 3, LBA30. Arrows indicate Oma87. The positions of standard molecular mass markers are shown on the left. The original was scanned with an Epson GT-8000 scanner and the Adobe Photoshop 2.5.1 LE program.

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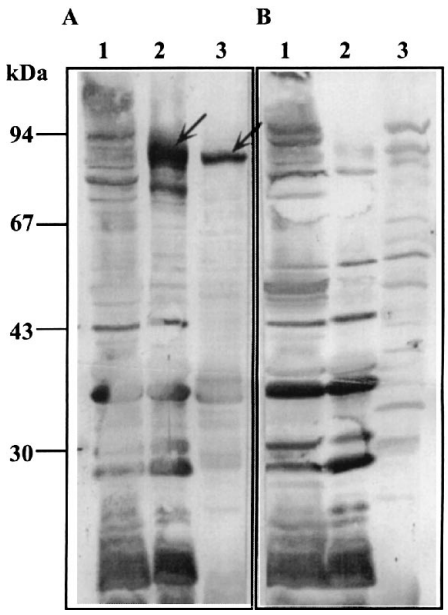

FIG. 8. Assessment of rabbit antisera used in passive protection studies. Whole-cell lysates of LBA30 (lanes 1), PBA1137 (lanes 2), and PBA100 (lanes 3) were immunostained with either antiserum from rabbits that received PBA1137 membrane fraction (CR3) (A) or antiserum from rabbits that received LBA30 membrane fraction (CR6) (B). Arrows indicate Oma87. The positions of standard molecular mass markers are shown on the left. The originals were scanned with an Epson GT-8000 scanner and the Adobe Photoshop 2.5.1 LE program.

alignment of Oma87 and D15 (Fig. 4), residues 247 to 345 and 730 to 743 constituted variable regions which corresponded to hydrophilic domains of Oma87. Therefore, these variable domains may represent unique epitopes, but such conclusions must await the results of detailed epitope mapping.

Immunological analysis of PBA100 and PBA1137 cell fractions localized the Oma87 protein predominantly to the outer membrane of the cell (Fig. 5). Evidence for surface localization of the protein was established by proteinase K treatment of the cells. Oma87 was not observed in whole cells treated with proteinase K prior to Western blotting with CR3 antiserum. Thus, the amino acid sequence and immunological analysis indicate that Oma87 is an OMP with surface-exposed regions.

Considering its significance as an important animal pathogen, most research on *P. multocida* has focused on identifying the immunogens that can elicit cross-protective immunity and thus be used in effective vaccines. Membrane antigens have been identified as potential immunogens and shown to elicit immune responses, but a limited number of membrane antigens of *P. multocida* have been identified and characterized (reviewed in reference 9). The 37.5-kDa protein H has been identified as a nonspecific bacterial porin and the major porin of *P. multocida* (7). Protein H is highly immunogenic, and antibodies directed against the protein are able to inhibit binding to respiratory mucosal surfaces (25, 26). A 50-kDa OMP with antiphagocytic activity was able to protect turkeys from lethal challenge (37), while a monoclonal antibody against a 37.5-kDa protein demonstrated protection against homologous challenge (24). The immunogenic 16-kDa P6-like OMP is expressed by all 16 serotypes of *P. multocida*. The protective capacity of the P6-like OMP has not yet been investigated, but because of its similarity to the *H. influenzae* P6 OMP, it has been suggested that the P6-like OMP may be a protective antigen (21). It has long been recognized that in vivo-grown *P. multocida*, and membrane-associated proteins of in vivo-grown cells, can induce cross-protective immunity (16, 31). Partially purified membrane proteins, with apparent masses of 39 kDa and 59 to 65 kDa, prepared from in vivo-grown cells have been associated with cross-protective immunity (30). Therefore, it is likely that cross-protective antigens are expressed in vivo. Both native and recombinant Oma87 were immunostained strongly by the chicken convalescent-phase antiserum CA101, implying that Oma87 is expressed during infection and is recognized by the humoral immune system. This may also be the case with *H. influenzae* D15 since convalescent-phase antisera from children have been shown to recognize this surface antigen (34). Western blot analysis with antibodies eluted from recombinant Oma87 showed that Oma87 was present in all *P. multocida* serotypes. However, whether Oma87 is present in all members of the *Pasteurellaceae* remains to be determined.

In studies with *H. influenzae*, affinity-purified anti-D15 antibodies were shown to be protective in the rat pup model (34). Accordingly, an investigation into the protective capacity of Oma87 was carried out with a mouse model using antiserum to recombinant Oma87. Mice that received CR3 antiserum were protected against a low challenge dose of PBA100, but this protection decreased when a higher challenge dose was used. The control antiserum, CR6, did not protect the mice against either homologous or heterologous challenges. The observed decrease in homologous protection with increased challenge dose may be due partly to low titers of circulating Oma87 antibodies. Although PBA101 expressed an Oma87 homolog, CR3 was unable to protect mice against the heterologous PBA101 strain. The lack of cross-protective immunity may be due to the different surface structure of PBA101, which produced increased amounts of capsule compared with PBA100 (unpublished observations), thus masking Oma87 and making it inaccessible to anti-Oma87 antibodies. Although PBA101 Oma87 reacted with the protective antiserum PM100, it is possible that specific protective epitopes may differ between the two strains. Nonreciprocal cross-protection has been observed with *P. multocida* avian strains, where serotype 3 protected against serotype 1 challenge but not vice-versa (16). It is thus possible that immunization with Oma87 from PBA101 may induce a cross-protective immune response. Active immunization studies with purified Oma87 in both mice and chickens will help to elucidate further the protective capacity of this protein and are currently being addressed.

The present study identified a surface-exposed membrane

TABLE 1. Protective capacity of either anti-Oma87 antiserum (CR3) or control antiserum (CR6) against lethal challenge with *P. multocida*

Antiserum used for passive immunization	Challenge strain (dose [ID_{50}])[a]	% Survival[b]
CR3	PBA100 (10^2)	100
CR3	PBA101 (10^2)	0
CR6	PBA100 (10^2)	0
CR6	PBA101 (10^2)	0
CR3	PBA100 (10^4)	20
CR3	PBA101 (10^4)	0
CR6	PBA100 (10^4)	0
CR6	PBA101 (10^4)	0
None (control)	PBA100 (10^2)	0
	PBA101 (10^2)	0

[a] The 50% infective doses (ID_{50}s) of PBA100 and PBA101 were 10^2 and 10 CFU, respectively.
[b] Groups of five mice were used for the immunization experiments.

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antigen of *P. multocida* which appears to be expressed in vivo. To our knowledge, this is the first recombinant antigen from an avian *P. multocida* strain to be used in a protection study and shown to be a potential protective membrane antigen.

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