JOURNAL OF BACTERIOLOGY, Dec. 1997, p. 7856–7864 Vol. 179, No. 24
0021-9193/97/$04.00+0

Cloning and Characterization of the Major Outer Membrane Protein Gene (*ompH*) of *Pasteurella multocida* X-73

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Received 25 June 1997/Accepted 15 September 1997

The major outer membrane protein (OmpH) of *Pasteurella multocida* X-73 was purified by selective extraction with detergents, followed by size exclusion chromatography. The planar lipid bilayer assay showed that OmpH has pore-forming function. The average single channel conductance in 1.0 M KCl was 0.62 nS. The gene (*ompH*) encoding OmpH has been isolated and sequenced by construction of a genomic library and PCR techniques. The coding region of this gene is 1,059 bp long. The predicted primary protein is composed of 353 amino acids, with a 20-amino-acid signal peptide. The mature protein is composed of 333 amino acids with a molecular mass of 36.665 kDa. The *ompH* gene encoding mature protein has been expressed in *Escherichia coli* by using a regulatable expression system. The *ompH* gene was distributed among 15 *P. multocida* serotypes and strain CU. Protection studies showed that OmpH was able to induce homologous protection in chickens. These findings demonstrate that OmpH is a protective outer membrane porin of strain X-73 and is conserved among *P. multocida* somatic serotypes.

Fowl cholera, caused by *Pasteurella multocida*, is an economically important infectious disease of chickens and turkeys. This disease has been poorly controlled and is still a severe problem in the poultry industry. Currently used vaccines, including inactivated and live vaccines, have their intrinsic disadvantages. The inactivated vaccines (bacterins) induce only serotype-specific immunity (there are 16 somatic serotypes). Attenuated live vaccines, for example, strains CU, M-9, and PM-1, can provide limited heterologous protection (33) but sometimes induce the disease (4, 7, 30, 38). Researchers are still looking for other effective vaccines against fowl cholera. For example, the in vivo-expressed "cross-protection factors" would be potential vaccine candidates (31–34, 42–44), but their expression is poorly understood.

Bacterial porins are channel-forming transmembrane proteins found in the outer membranes of gram-negative bacteria. They function as molecular sieves to allow the diffusion of small hydrophilic solutes through the outer membrane and also serve as receptors for bacteriophages and bacteriocins (15). Porins are highly immunogenic, exposing epitopes on the bacterial surface. They are generally conserved in a bacterial species or even in a bacterial family in that they have high homology in primary amino acid sequence and secondary structure and are antigenically related (16, 35). These properties make porins attractive vaccine candidates for induction of homologous and heterologous immunity against gram-negative bacterial infections (10, 22, 25, 26, 39).

Protein H, or porin H, is the major outer membrane protein in the envelope of *P. multocida* (20, 21). This protein has been purified and characterized as a porin because it is structurally and functionally related to the superfamily of porins of gram-negative bacteria (8, 20). In native conformation, porin H is a homotrimer, stable in sodium dodecyl sulfate (SDS) at room temperature, and is dissociated into monomers upon boiling. The molecular masses of denatured monomers range between 34 and 42 kDa depending on the serotype and the electrophoretic system used for analysis (8, 19, 20). The N-terminal amino acid sequence of porin H has been determined for serotype D2 (8). This N-terminal sequence is almost identical to that of so-called cross-protection factors OMP 179 and OMP 153 from strain P-1059 (serotype 3), which are in the high-molecular-mass range (42–44). The relationship between porin H and the cross-protection factors is unclear. Manoha et al. reported that they might have cloned a portion of the porin H gene of strain 9222 (serotype D2) by genomic library construction and an immunological screening method (23). But in a later study they found that the cloned genes were not the porin H gene but putative *skp* and *firA* genes, which showed high homology to the *skp* and *firA* genes of *Escherichia coli* (9). The reasons for the difficulty in cloning this biologically important gene have been unclear.

In this report, we describe the cloning and characterization of the major outer membrane porin gene of *P. multocida* X-73. We have designated this gene *ompH* and the encoded major outer membrane protein OmpH.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains or serotypes of *P. multocida* used in this research were X-73 (serotype 1), P-1059 (serotype 3), P-1662 (serotype 4), P-1702 (serotype 5), the type strains of serotypes 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, and 16, and the CU vaccine strain (serotype 3,4). All isolates were obtained from the National Animal Disease Center (Ames, Iowa). *E. coli* XL1-Blue MRF′ was obtained from Stratagene (La Jolla, Calif.). pUC18 was obtained from Boehringer Mannheim (Indianapolis, Ind.). pNOTA/T7 and the relevant Prime PCR Cloner system was obtained from 5 Prime→3 Prime, Inc. (Boulder, Colo.). The pQE30 and pQE32 expression vector kit was obtained from Qiagen, Inc. (Chatsworth, Calif.).

Purification of major outer membrane protein OmpH of X-73. The major outer membrane protein of *P. multocida* X-73 was purified according to the

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method described by Chevalier et al. (8). Briefly, the bacteria were cultured in brain heart infusion broth at 37°C overnight. The bacteria were then harvested and washed three times with phosphate-buffered saline (PBS). The washed bacteria were sonicated and then incubated with 2% sodium *N*-lauroyl sarcosinate in 50 mM Tris-HCl buffer (pH 7.4) and centrifuged at 206,000 × *g* at 16°C for 1 h. This step was repeated once, and the insoluble material was dispersed in 2% SDS, 0.5 M NaCl, and 5 mM EDTA in 50 mM Tris-HCl buffer (pH 7.4) by stirring at 37°C for 2 h. The supernatant was recovered by centrifugation at 206,000 × *g* at 25°C for 1 h. The supernatant was then applied on a 60-by-600 Superdex 200 column (Pharmacia Biotech, Piscataway, N.J.), equilibrated, and eluted with 50 mM sodium phosphate buffer (pH 7.4) containing 0.1% SDS and 0.3 M NaCl. This procedure was performed at 25°C to prevent SDS precipitation. Elution of the protein was monitored by UV absorption at 280 nm. The fractions containing OmpH were identified by SDS-polyacrylamide gel electrophoresis (PAGE) and pooled.

Quantitation of protein and KDO. Protein was quantitated by the method of Lowry et al. (18) with bovine serum albumin as a standard. 2-Keto-3-deoxyoctonate (KDO) was determined as described by Hanson and Phillips (14) by using commercial KDO (Sigma, St. Louis, Mo.) as a standard.

N-terminal amino acid sequencing. N-terminal sequencing of the purified X-73 outer membrane protein and recombinant protein were performed by the Edman method on a Procise 494 protein sequencing system (Applied Biosystems, Foster City, Calif.).

Oligonucleotide synthesis. Oligonucleotides were synthesized by the Molecular Genetics Instrumentation Facility of The University of Georgia (Athens, Georgia) and Retrogen Inc. (San Diego, Calif.). Deoxyinosine was also utilized for primers synthesized according to the N-terminal amino acid sequence of OmpH (see Table 1).

Construction of a genomic DNA library and extraction of plasmids. Genomic DNA of X-73 was isolated with the GNOME DNA isolation kit (Bio 101, Inc., La Jolla, Calif.). The genomic DNA was partially digested with *Sau*3AI or digested to completion with *Taq*I. Fragments between 2 and 20 kb of partially digested genomic DNA (fractionated on an agarose gel) or the fragments of completely digested genomic DNA were ligated into pUC18 which had been digested with *Bam*HI or *Acc*I and dephosphorylated with alkaline phosphatase. The ligations were transformed into competent *E. coli* XL1-Blue MRF′ cells by electroporation with a Gene Pulser (Bio-Rad Laboratories, Hercules, Calif.). The transformants were plated on Luria-Bertani medium containing 200 μg of ampicillin per ml. Libraries were screened by colony hybridization with a digoxigenin-labeled oligonucleotide as described in the manufacturer's instructions (Genius System User's Guide; Boehringer Mannheim), or all of the clones on the plates were harvested and the plasmids were extracted by using the PERFECT Prep kit (5 Prime→3 Prime, Inc.).

Amplification of DNA by PCR. A portion of the *ompH* gene was amplified from a genomic DNA library by PCR as follows. The PCR mixture consisted of 50 ng of genomic library plasmid mixture, 100 pmol of degenerate primers synthesized according to the N-terminal amino acid sequence of OmpH (primers A and B; Table 1), 100 pmol of M13 sequencing primers (primers C and D; Table 1), 0.1 mM deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, and 1.25 U of *Taq* DNA polymerase (Boehringer Mannheim) in 50 μl of reaction buffer. The amplification reaction included 1 cycle at 94°C for 2 min; 35 cycles at 94°C for 15 s, 55°C for 1 min, and 72°C for 1 min; and 1 cycle at 72°C for 10 min. The reactions were carried out on a Gene Amp PCR System 9600 (Perkin-Elmer Cetus Inc., Norwalk, Conn.). The whole *ompH* gene was amplified by PCR as follows. The PCR mixture consisted of 10 ng of genomic X-73 DNA, 30 pmol of *ompH* gene N terminus primers, and 30 pmol of *porH* gene downstream primers; other components are the same as described above. The amplification reaction included 1 cycle at 94°C for 5 min; 35 cycles at 94°C for 15 s, 55°C for 1 min, and 72°C for 1 min; and 1 cycle at 72°C for 10 min.

Inverse PCR. Inverse PCRs were carried out according to the method of Ochman et al. (29). Genomic DNA of X-73 was digested to completion with *Sau*3AI or *Hin*dIII. The digested DNAs were purified and diluted to 10 ng/μl. Self-ligations were carried out at 16°C overnight. For PCR, two DNA polymerase systems were used. *Taq* DNA polymerase was used for DNA amplification of self-ligation of *Sau*3AI-digested DNA. The Expand long-template PCR system (Boehringer Mannheim) was used for DNA amplification of self-ligation of *Hin*dIII-digested DNA. The PCR cycle for *Taq* DNA polymerase was 1 cycle at 94°C for 2 min; 40 cycles at 94°C for 10 s, 60°C for 30 s, and 70°C for 2 min; and 1 cycle at 72°C for 10 min. The PCR cycle for the Expand long-template PCR system was 1 cycle at 94°C for 2 min; 40 cycles at 94°C for 10 s, 60°C for 30 s, and 68°C for 4 min; and 1 cycle at 68°C for 10 min.

Subcloning of PCR products. PCR products were cloned into plasmid pNOTA/T7 for subsequent sequencing according to the manufacturer's instructions (5 Prime→3 Prime, Inc.). PCR-amplified whole *ompH* genes were subcloned into the pQE30 and pQE32 expression vector system for expression analysis according to the manufacturer's instructions (Qiagen).

DNA sequence determination and analysis. The PCR products and subcloned PCR inserts in plasmids were sequenced by the dideoxy chain termination method (36) with an Applied Biosystems model 373A, version 2.1.0, DNA sequencer. Sequence analysis was conducted with Hitachi DNAsis Pro 3.0 software (Hitachi Software Engineering Co., Ltd., San Bruno, Calif.) and the Gene Construction Kit (Textco, Inc., West Lebanon, N.H.). Sequence similarity searches were performed at the National Center for Biotechnology Information with the BLAST network service (1).

Southern blots and dot blots. Southern blots were carried out according to Genius System User's Guide (Boehringer Mannheim). The probe used was oligonucleotide N (Table 1), 3′ end labeled with digoxigenin according to the manufacturer's instructions (Boehringer Mannheim). Hybridization was carried out under high stringency (determined by washing at 42°C in 0.1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]). DNA dot blotting was carried out as follows. Bacterial DNA was diluted to 200 μg/ml in Tris-Cl-EDTA buffer, heated to 100°C for 10 min, and chilled immediately on ice. A 1-μl (0.2 μg) DNA dilution was spotted onto a positively charged nylon membrane (Boehringer Mannheim). The DNA was fixed by baking at 120°C for 30 min. The probe used was a PCR-amplified *ompH* gene sequence from X-73 labeled with digoxigenin by the Genius 2 DNA kit. Hybridization and detection were performed as described by the manufacturer (Boehringer Mannheim). Hybridization was carried out under high stringency (determined by washing at 65°C in 0.5× SSC).

Expression of *ompH* in *E. coli* and purification of the recombinant protein. The *ompH* gene of X-73, encoding primary and mature proteins, was amplified, respectively, from genomic X-73 DNA with two pairs of primers corresponding to the N-terminal and downstream sequences of the *ompH* gene (primer G paired with primer I for primary protein and primer H paired with primer I for mature protein) (see Table 1 and Fig. 3). These two PCR products were ligated into the expression vectors pQE30 and pQE32 and transformed into competent *E. coli* XL1-Blue MRF′. (pQE30 and pQE32 expression vectors have an isopropyl-β-D-thiogalactopyranoside [IPTG]-regulated promoter and a T5 promoter containing two *lac* operator sequences, followed by the multiple cloning site with a six-histidine tag. Under the induction of IPTG, the six-histidine tag and the inserted gene were expressed as a fusion protein. The fusion protein can be easily purified by affinity chromatography with Ni-nitrilotriacetic acid resin [see the Qiagen QIAexpressionist instructions]). Transformants were plated on Luria-Bertani plates containing 200 μg of ampicillin per ml. The plasmids in the transformants were extracted, and the inserts in the plasmids were sequenced to confirm that they contained the right sequence of the *ompH* gene. Transformants containing *ompH* were cultured in SOB broth with or without IPTG. The recombinant proteins were purified according to the manufacturer's instructions (Qiagen).

SDS-PAGE and Western blots. Samples were analyzed on a polyacrylamide gel according to Laemmli's method (17). Bacterial whole-cell lysates (40 μg per well) and purified native X-73 OmpH and recombinant protein (4.5 μg per well) were applied to 10% polyacrylamide gels and electrophoresed at 20 mA. The gels were stained with Coomassie blue R-250 for detection of proteins. For Western blots, the proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories) at 80 V for 2 h. Nitrocellulose membranes were then incubated with primary antiserum for 2 h followed by washes in PBS three times. The membranes were then incubated with a 1:500 dilution of horseradish peroxidase-conjugated anti-chicken immunoglobulin G (Sigma) for 1 h, followed by washes in PBS three times again. Antigens on membranes were visualized by incubation with 3,3′-diaminobenzidine (DAB) and urea hydrogen peroxide solution prepared with a fast DAB tablet (Sigma).

Antibody production. Antisera against bacterin of *P. multocida* X-73 were prepared as described previously (42, 43). Antisera against native X-73 OmpH and recombinant protein from *E. coli* were prepared as follows. Purified native X-73 OmpH and recombinant protein were emulsified in complete Freund adjuvant. The ratio of aqueous phase to adjuvant was 1:3. The preparation was injected, at 0.5 ml (100 μg of protein) per bird, twice intramuscularly (at 5 and 8 weeks of age) in specific-pathogen-free (SPF) chickens. Blood was collected 14 days after the second injection. All of the above antisera were absorbed with *E. coli* XL1-Blue MRF′ whole-cell lysates before being used on Western blots.

Enzyme-linked immunosorbent assay (ELISA). Immunoplates (Nunc VWR Scientific, Bridgeport, N.J.) were coated at 4°C overnight with 100 ng of the following antigens: purified X-73 OmpH, recombinant protein, and X-73 whole cell lysate in borate buffer (pH 9.5). The plates were washed three times with 0.01 M PBS containing 0.05% Tween 20 (pH 7.2), followed by the addition of 200 μl of blocking buffer (PBS containing 1% bovine serum albumin), and were incubated at room temperature for 30 min. After the plates were washed, 50 μl of antisera serially diluted with blocking buffer was added and the plates were incubated at room temperature for 30 min. After a further washing, 50 μl of 1:5,000 diluted rabbit anti-chicken immunoglobulin G conjugated to horseradish peroxidase (Zymed Laboratories, Inc., South San Francisco, Calif.) was added and the plates were incubated at room temperature for 1 h. For color development, 100 μl of 3,3′,5,5′-tetramethylbenzidine substrate was added and the plates were incubated for 30 min. Then, 100 μl of a 0.25% solution of hydrofluoric acid was added to stop the reaction. Absorbance was read at a wavelength of 630 nm with an ELISA reader (MR650; Dynatech Laboratories, Inc., Alexandria, Va.).

Functional assays with planar lipid bilayers. The pore-forming activities of purified X-73 outer membrane protein OmpH and recombinant protein were examined by using planar lipid bilayers (2, 3, 12). Lipid bilayers made from 1.5% oxidized cholesterol in *n*-decane were formed across a 0.1-mm^2 hole separating two compartments of a Teflon chamber containing 1.0 M KCl. Electrodes were implanted in each compartment, one connected to a voltage source and one to a current amplifier and chart recorder, with the output monitored on an oscil-

loscope. The protein samples were highly diluted in 0.1% Triton X-100, and approximately 5 ng of the protein was added to one compartment. A voltage of 50 mV was applied across the lipid bilayer. Increases in conductance were recorded, and average single-channel conductances were calculated.

Protection studies in chickens. Purified X-73 outer membrane protein OmpH and recombinant protein from *E. coli* were mixed with monophosphoryl lipid A (Sigma), 0.25 mg/ml. The preparations were used for vaccination in SPF chickens. The birds were divided into five groups with 10 birds per group. Group 1 and group 2 chickens were injected intramuscularly with purified native X-73 OmpH and recombinant protein preparation, respectively, at 100 μg of protein (0.5 ml) per bird. Group 3 chickens were injected intramuscularly with 100 μg of native OmpH treated with protease (Boehringer Mannheim) per bird. Group 4 chickens were injected intramuscularly with *P. multocida* X-73 bacterin prepared as described previously (42, 43). Group 5 chickens were not vaccinated. The birds were vaccinated twice (at 5 and 8 weeks old). Fourteen days after the second vaccination, a blood sample was taken from each bird and the birds were challenged with 100 CFU of *P. multocida* X-73. The birds were observed for 10 days after challenge, and mortalities were recorded.

Nucleotide sequence accession number. The DNA sequence of the *ompH* gene has been deposited in GenBank under accession no. U50907.

RESULTS

Purification and amino acid sequencing of X-73 major outer membrane protein OmpH. The X-73 major outer membrane protein was purified by detergent treatment of the cell envelope and size exclusion chromatography. The purified outer membrane protein still contained a trace amount of lipopolysaccharides (LPSs), indicated by the detection of 0.418 μg of KDO mg of protein^{-1}. This indicated that OmpH tends to associate with LPS, as has been described for other porins (11, 37). The whole-cell lysate of X-73 and purified OmpH were analyzed by PAGE and Western blotting (Fig. 1, lanes 1 to 4). The protein samples dissolved in loading buffer were treated with incubation at 37°C for 30 min or boiled at 100°C for 10 min before being loaded on the gel. A heat-modifiable property was observed for the major outer membrane protein OmpH (Fig. 1A and B, lanes 1 to 4; the arrows indicate the positions of OmpH monomer). As shown in Fig. 1, the major outer membrane protein band (OmpH monomer) disappeared when the sample was not boiled, but a ladder of high-molecular-mass protein bands between 76 and 210 kDa appeared at the top region of the gel. The same phenomena occurred with the purified outer membrane protein (Fig. 1A and B, lanes 3 and 4). In SDS-PAGE analysis, the boiled purified outer membrane protein contained a major band with a molecular mass of about 37 kDa, some faint bands at the higher-molecular-mass position, and a faint band with a molecular mass of about 35 kDa (Fig. 1A, lane 4). Comparing this with the Western blot assay, in which these faint bands were able to react to the antiserum against recombinant OmpH (Fig. 1C, lane 4), it could be suggested that the faint bands at the higher-molecular-mass position probably were the insolubilized trimers and oligomers or aggregates of denatured monomers of OmpH and that the faint 35-kDa band was probably the undenatured monomers, although the possibility that they were contaminants or degraded OmpHs could not be completely excluded. Similar phenomena also occurred in previous studies of putative *P. multocida* porins (8, 19, 24).

For the unboiled purified outer membrane protein, besides the ladder of high-molecular-mass protein bands between 76 and 210 kDa, there was also a trace amount of monomers (Fig. 1, lane 3). This indicated that a small amount of trimeric or oligomeric form of OmpH was denatured during purification. The ladder of high-molecular-mass protein bands probably represented OmpH trimers or oligomers consisting of different numbers of monomers. They might also represent trimers associating with different amounts of LPS. Similar phenomena of the unboiled porin appearing as a ladder of high-molecular-mass protein bands on polyacrylamide gels were also reported

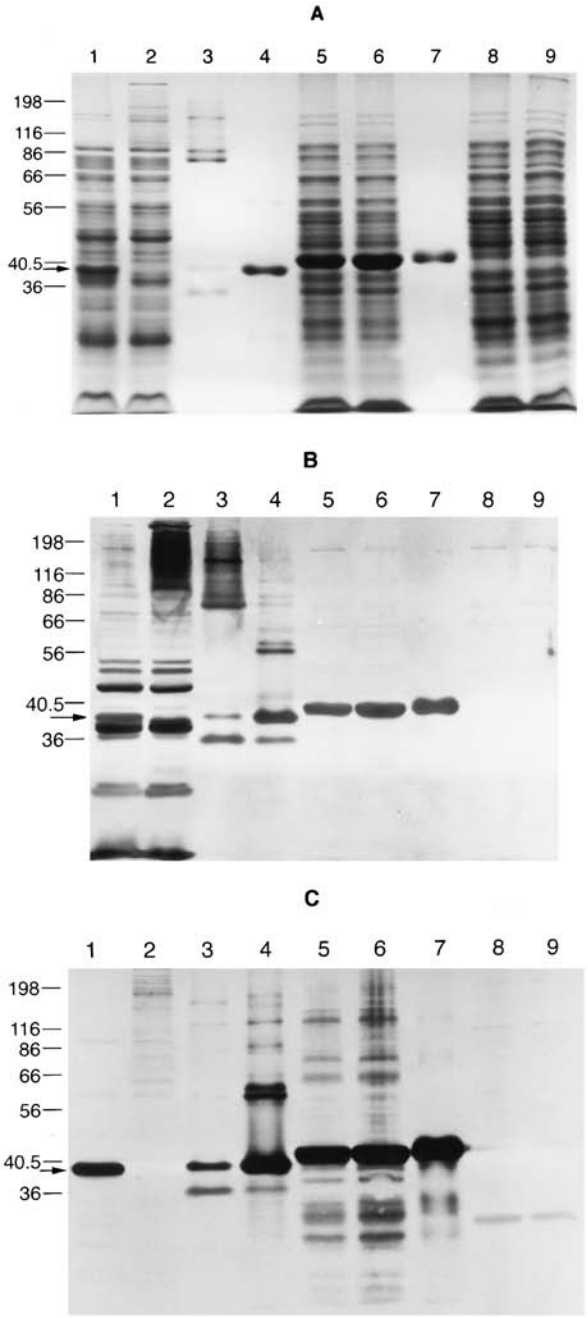

FIG. 1. SDS-PAGE and Western blot assay of native X-73 OmpH and recombinant protein of *E. coli*. (A) SDS-PAGE of X-73 and *E. coli* whole-cell lysates and purified native OmpH and recombinant protein on a 10% gel stained with Coomassie blue. (B) Western blots of the gel in panel A probed with antiserum against X-73 bacterin. (C) Western blots of the gel in panel A probed with antiserum against recombinant protein. Lanes: 1, boiled X-73 whole-cell lysate; 2, X-73 whole-cell lysate incubated at 37°C; 3, purified X-73 OmpH incubated at 37°C; 4, boiled purified X-73 OmpH; 5, whole-cell lysate of XL1-Blue MRF′ harboring pJYH1 (IPTG uninduced); 6, whole-cell lysate of XL1-Blue MRF′ harboring pJYH1 (IPTG induced for 10 h); 7, purified recombinant protein; 8, whole-cell lysate of XL1-Blue MRF′ harboring pQE32 without insert (IPTG uninduced); 9, whole-cell lysate of XL1-Blue MRF′ harboring pQE32 without insert (IPTG induced for 10 h). Samples in lanes 5 to 9 were boiled before being loaded. Numbers on the left indicate the positions of molecular mass standards (in kilodaltons).

TABLE 1. Oligonucleotides and primers used in this study

Oligonucleotide or primer	Sequence	Position[a]
Oligonucleotide N	5′-AC(T/C/A/G)GT(T/C/A/G)TA(T/C)AA (T/C)CA(A/G)GA(T/C)GG-3′	455–475
Primers		
A	5′-GTITA(T/C)AA(T/C)CA(A/G)GA(T/C) GGIAC-3′	459–478
B	5′-AA(T/C)CA(A/G)GA(T/C)GGIACIAA (A/G)GT-3′	465–484
C	5′-TGTAAAACGACGGCCAGT-3′	M13 forward
D	5′-AGCGGATAACAATTTCACACAGGA-3′	M13 reverse
E	5′-GCTTAAGCCTTCGCCTAAATC-3′	604–584
F	5′-TTTGGTGGTGCGTATGTCTTCT-3′	905–926
G[b]	5′-TCAACTATGAAAAAGACAATCGTAG-3′	389–410
H[b]	5′-TCACAGCAACAGTTTACAATCAAGA-3′	450–471
I[b]	5′-CTAGATCCATTCCTTGCAACATATT-3′	1,604–1,584

[a] Position in the X-73 *ompH* gene (Fig. 3), given in base pairs.
[b] Three extra bases were added at the 5′ end to facilitate subcloning of the PCR products.

for other putative *P. multocida* porins (24). It was noted that in the Western blot assay, the antiserum against recombinant protein did not react to the unboiled native trimeric or oligomeric proteins of X-73 OmpH (Fig. 1C, lane 3). This indicated that the recombinant protein was denatured and did not contain the conformational epitopes of native OmpH.

The amino acid sequence of the purified OmpH, determined over 20 residues, was A-T-V-Y-N-Q-D-G-T-K-V-D-V-N-G-S-L-R-X-I. This N-terminal amino acid sequence was almost identical to that of previously reported porin H from *P. multocida* serotype D2 except for differences at positions 13 and 17 (8). The N-terminal amino acid sequence was also very similar to that of other reported putative porins of *P. multocida* (19, 24).

Design of synthetic oligonucleotides and hybridization with *P. multocida* DNA. Three degenerate oligonucleotides, oligonucleotide N, primer A, and primer B, were synthesized according to the N-terminal amino acid sequence of OmpH and the previously reported porin H. Primer C and primer D were M13 forward and reverse sequencing primers derived from the pUC18 sequence. Other primers, primers E to I, were also synthesized according to *ompH* gene sequence. Primers A to I were used for PCR amplifications. The sequences and the positions of the primers in the subsequently cloned *ompH* gene sequence are shown in Table 1.

Oligonucleotide N was used for hybridization. DNA dot blotting with high stringency showed that it hybridized with 15 somatic serotypes and the CU vaccine strain (data not shown). Southern blotting of X-73 DNA showed that it hybridized with a single restriction fragment (Fig. 2). The approximate sizes of the hybridized fragments were as follows: *Hin*dIII, 3.8 kb; *Sau*3AI, 1.4 kb; and *Taq*I, 0.66 kb. These results also indicated that there was a single copy of *ompH* in the chromosome.

Cloning and sequencing of *ompH* gene fragments of X-73. Initially, libraries were constructed with *Sau*3AI-partially-digested X-73 genomic DNA and screened with a probe of oligonucleotide N. The screening results were repeatedly negative regardless of changing vectors and *E. coli* strains, and the backgrounds were also high. Subcloning of gel-extracted oligonucleotide N-hybridized *Hin*dIII and *Sau*3AI fragments was also negative. The negative results suggested that *P. multocida* OmpH is lethal for *E. coli*. This suggested that the smaller oligonucleotide N-hybridized fragments in the *Taq*I digest of X-73 DNA, which presumably did not contain a complete *ompH* gene, would be suitable candidates for cloning. A new library was constructed with *Taq*I-completely-digested X-73 genomic DNA. The plasmids of the harvested library clones (mixture) were extracted. PCRs in which the whole library plasmid mixture was used as a template, primer A or primer B was used as the 5′ primer, and primer C (M13 forward sequencing primer) or primer D (M13 reverse sequencing primer) was used as the 3′ primer were carried out (Table 1; also see Materials and Methods). An obvious PCR product of about 640 bp was obtained when primer A or primer B was paired with primer D (data not shown). These PCR products were purified from the gels for sequencing. The DNA sequences were determined by both direct sequencing of the PCR products and subcloning of the PCR products into pNOTA/T7 plasmids and sequencing. A clear 540-bp sequence was obtained. The deduced N-terminal amino acid sequence matched that of X-73 OmpH determined by amino acid sequencing. Based on this sequence, primer E and primer F, corresponding to the sequence near both ends of the 540-bp fragment, were synthesized (Table 1). Southern blotting showed that both primer E and primer F hybridized with the *Sau*3AI and *Hin*dIII fragments shown in Fig. 2 (data not shown). Therefore these *Sau*3AI and *Hin*dIII fragments contained the flanking sequence of the 540-bp fragment. *Sau*3AI and *Hin*dIII were used to digest X-73 genomic DNA to completion. After self-ligation, the inverse PCRs were carried out. PCR products were analyzed and sequenced as described above. By comparing and analyzing the sequences of the initial PCR products and the subsequent inverse PCR products, the complete X-73 *ompH* gene sequence, including the upstream and downstream regulatory regions, was obtained (Fig. 3).

DNA sequence analysis and hybridization of the *ompH* gene with *P. multocida* DNA. The coding region of *ompH* is 1,059 bp long. The predicted primary protein is composed of 353 amino acids, with a 20-amino-acid signal peptide. The signal peptide

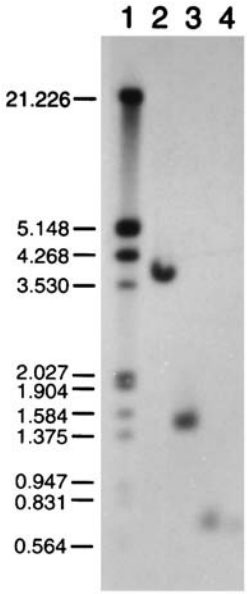

FIG. 2. Southern blot of complete restriction enzyme digests of X-73 genomic DNA probed with oligonucleotide N. Lanes: 1, molecular size markers; 2, *Hin*dIII digests; 3, *Sau*3AI digests; 4, *Taq*I digests. Numbers on the left indicate molecular sizes, in kilobases.

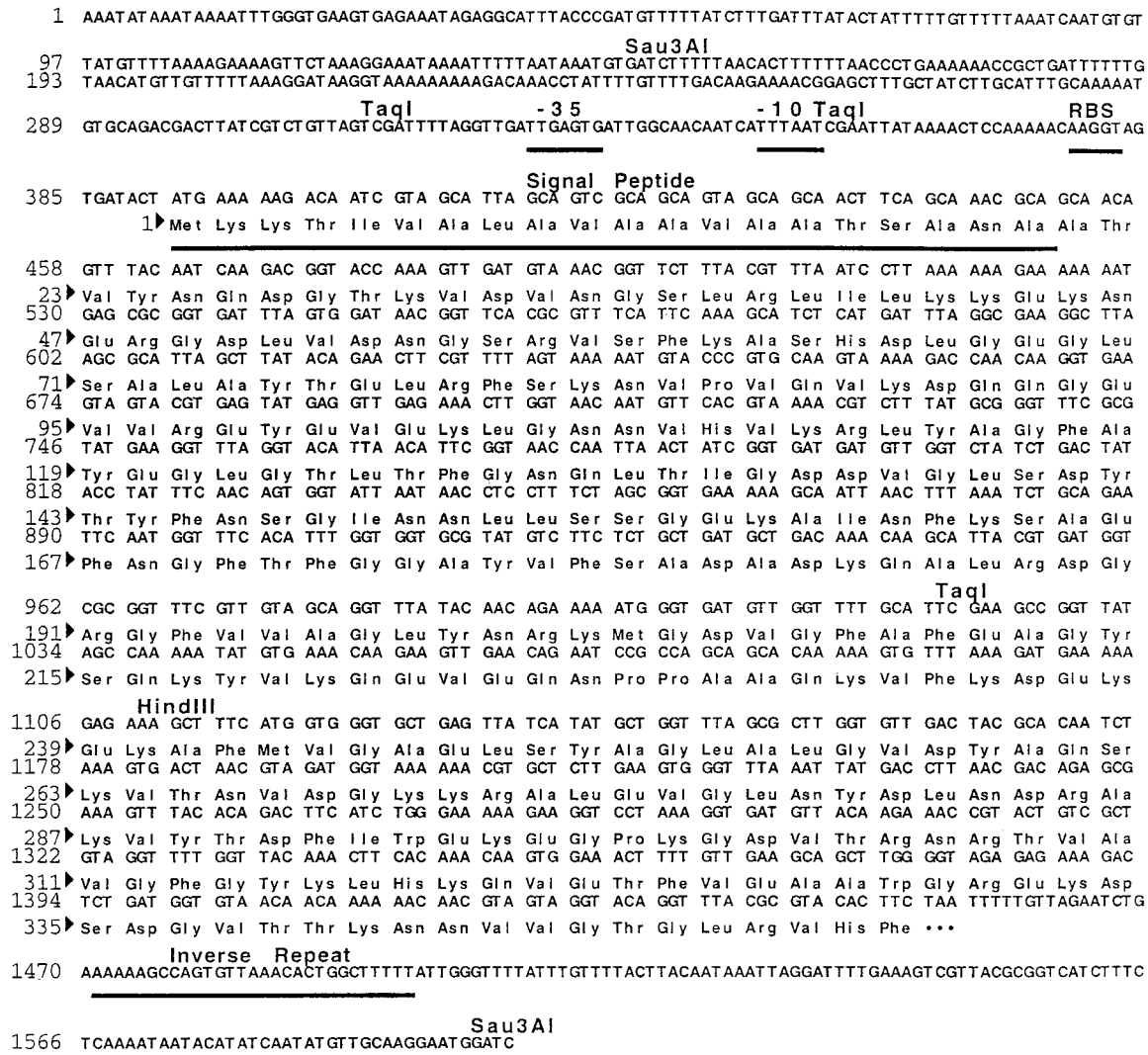

FIG. 3. DNA sequence of the *ompH* gene of X-73. The predicted amino acid sequence is shown under the DNA sequence, with the signal peptide indicated. The putative promoter sequences (−35 and −10), the ribosome binding site (RBS), and the inverse repeat are shown, as are the *Hin*dIII, *Sau*3AI, and *Taq*I sites.

has the common characteristics associated with such sequences (41), a stretch of hydrophobic amino acids and an Ala-X-Ala cleavage site. The mature protein contains 333 amino acids with a predicted molecular mass of 36.665 kDa, which was very close to the molecular mass of the native OmpH determined electrophoretically. There are also putative −35 and −10 promoter sequences which are similar to the consensus for these sequences in *E. coli*. These regions may be the reason that the whole *ompH* gene could not be cloned in *E. coli*, because this promoter sequence would function in *E. coli* and result in the unregulated expression of toxic OmpH. There is also a Shine-Dalgarno ribosome binding site just 9 bp before an ATG start codon and an inverse repeat as a terminator after a TAA stop codon. The predicted N-terminal amino acid sequence matches that of OmpH determined by amino acid sequencing. A sequence similarity search in the GenBank database revealed that the *ompH* gene and the predicted amino acid sequence show similarities to other bacterial porins, especially to *Haemophilus influenzae* porin P2 (38% identity in the amino acid sequence) (Fig. 4). The amino acid composition of OmpH is typical of nonspecific bacterial porins in its highly negative hydropathy index, high glycine content, low proline content, and lack of cysteine (data not shown).

Fifteen somatic serotypes of *P. multocida* and the CU vaccine strain were analyzed to determine the homology and distribution of the *ompH* gene. The labeled *ompH* gene sequence hybridized, under high stringency, with genomic DNAs from 15 somatic serotypes as well as the vaccine strain CU (data not shown).

Expression of the *ompH* gene in *E. coli*. Transformation of recombinant pQE30, which contained the *ompH* gene for the primary protein-containing signal peptide, was repeatedly unsuccessful, or only a few colonies were obtained. But the plasmids in all of these colonies contained a truncated or deleted *ompH* gene after sequence determination analysis. This further supports the idea that even a small amount of *ompH* gene-expressed protein is lethal for *E. coli*. Transformation of *E. coli* with recombinant pQE32, which contained the *ompH* gene for the mature protein without a signal peptide, resulted in about 2,000 colonies. Five colonies were randomly picked for sequence confirmation of the insert in the plasmids. All of them contained the right sequence. One colony was chosen for fur-

```
  1 MKKTIVALAVAAVAATSA-NAATVYNQDGTKVDVNGSLRL-ILKKEK---      50
    |||| || | | || || ||| ||| |||| | | | |
  1 MKKTLAALIVGAFAA-SAANAAVVYNNEGTKVELGG--RVSIIA-EQSTS      50

 51 NERGD-------LVDN-GSRVSF--KASHDLGEGLSALAYTELRFSKNVP     100
    | | |       | | ||| | | | || | || | | || |
 51 N-RKDQKHQHGSLR-NQGSR--FNIKVTHNLGDGYYALGYYETRFI-N--     100

101 VQVKDQQGEVVREYEVEKL-GNNVHV---KRL-YAGFAY-EGLGTLTFGN     150
      || | | | | | | ||| | || |||
101 ---KDIDGN---E----KNIGSGFGSITTK-LAYAGLGNKE-LGEATFGL     150

151 QLTIGDDVGLS---DYTYFNSG-IN-NLLSSG--EK-AINF--KSAEFNG     200
    | || | | | | | | | | | | | | | |
151 QKTIADKI--STAEDKEY---GVIEKN--SYIPTEGNAIAYTYKGIE--G     200

201 FTFGGA-YVF-----SADA---D-KQ--ALRDGRGFV-VAGL-Y--NRKM     250
     | | | |||     | |    | |   |       | | |  |  |
201 LTLG-ASYVFGGRNFS-DYEITDGKVSNA-------VQV-GAKYDANNI-     250

251 GDV-GFAFEAGYSQKYVK-QEVEQNPPAA-QKVFKDEKEKAFMVGAELSY     300
      | |||   |    | | |   |   |  | |       |    | | |
251 --VAGFAY--GRTN-Y-KAQ---Q---AKTQQVNG-----AL---ATLGY     300

301 A----GLALGV-D--YAQSKVT-NV-DGK--KRALEV--GLNY----D--     350
        || |   |  ||  | | |  | |  ||   |  |  |    |
301 HFDDLGL-LISLDSGYA--K-TKNKAD-KHEKRYF-VSPGFQYELMEDTN     350

351 L--NDRAKVYTDFIWEKE-GPKGDVTRNRTVAVGFGY--KLHKQVETFVE     400
    |  |   | |   |     | |  | |    || ||   |||||| |  |
351 LYGNL--K-YER-INSVDQGEK--V-REH--AVLFGIDHKLHKQVLTYIE     400

401 -A-AWGREKDSDGVT--T-KNNVVGTGLRVHF.................     450
     | |  |  |  | |  | |   || |||| |
401 GAYARTRTNDK-GKTEKTEKEKSVGVGLRVYF.................     450
```

FIG. 4. Comparison of the amino acid sequences of *P. multocida* OmpH (top lines) and *H. influenzae* porin P2 (bottom lines). Identity (vertical lines) is indicated. The sequence of porin P2 is from GenBank, accession no. X73393.

ther expression analysis. The plasmid in this colony was designated pJYH1. The recombinant protein was purified, and the N terminus was sequenced to confirm that the recombinant protein contained the N-terminal amino acid sequence of OmpH (data not shown). The recombinant protein has 13 amino acids fused at the N terminus of OmpH. The recombinant OmpH expressed in *E. coli* was analyzed and detected by PAGE and/or Western blots (Fig. 1, lanes 5 to 7). It was interesting that both induced and uninduced *E. coli* harboring pJYH1 produced the recombinant OmpH. This indicated that the T5 promoter in pJYH1 was not tightly controlled and that leaking expression occurred. This also indicated that the mature recombinant OmpH was not lethal for *E. coli*. The nonlethality of mature recombinant OmpH was further proven by the fact that even overnight induction by IPTG did not affect the viability of *E. coli* (data not shown). The fusion recombinant protein had a molecular mass of about 40 kDa and was the most abundant protein produced by *E. coli* (Fig. 1).

In Western blotting analysis, three antisera—antiserum against X-73 bacterin, antiserum against purified native OmpH, and antiserum against recombinant OmpH—were applied to detect the recombinant protein. All of the antisera reacted with the recombinant protein, and the reaction patterns were the same (Fig. 1B and C; data for antiserum against purified native OmpH not shown). Antiserum against recombinant OmpH of X-73 also reacted to protein bands with molecular masses from 34 to 37 kDa in 15 somatic serotypes of *P. multocida* as well as the CU vaccine strain (Fig. 5).

Single-channel conductance of X-73 OmpH and recombinant protein. In order to obtain a single insertion of the porins into the bilayer, the protein samples were highly diluted and added to one compartment of the chamber. A voltage of 50 mV was applied. When the purified X-73 OmpH was added, stepwise increases in membrane conductance were observed. This was attributed to the insertion of OmpH into the bilayer and caused the cross-membrane flow of ions in the aqueous phase. The conductance events were amplified through a current amplifier and recorded by a chart recorder (Fig. 6). The observed staircase pattern of conductance increase is typical of porins (2, 3, 12). The distribution of single-channel increments in conductance caused by X-73 OmpH in 1.0 M KCl is shown in Fig. 7. The average conductance for single channels was 0.62 nS. No conductance increase was observed for recombinant protein (data not shown). Circular dichroism (CD) spectroscopy was also conducted for comparison of the CD spectra between native OmpH and recombinant protein. There were significant differences in their CD spectra, which indicates that the native OmpH contains a large amount of beta sheets, but the recombinant protein mainly contains irregular structures (data not shown). These data explain the importance of native conformation of porin in pore-forming activity in the lipid bilayer, as was described previously by Chevalier et al. (8).

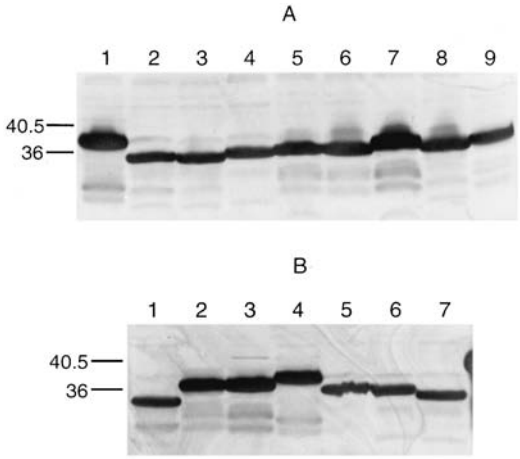

FIG. 5. Western blots of whole-cell lysates of 15 *P. multocida* serotypes and the CU strain probed with antiserum against recombinant OmpH. Lanes in panel A: 1, X-73; 2, P-1059; 3, P-1662; 4, P-1702; 5, serotype 6; 6, serotype 7; 7, serotype 8; 8, serotype 9; 9, serotype 10. Lanes in panel B: 1, serotype 11; 2, serotype 12; 3, serotype 13; 4, serotype 14; 5, serotype 15; 6, serotype 16; 7, strain CU. Numbers on the left indicate positions of molecular mass standards (in kilodaltons).

Protection studies. Five groups of SPF birds were used for protection studies using the purified native X-73 OmpH and the recombinant OmpH. *P. multocida* X-73 bacterin was used as a positive control, and the nonvaccinated groups of birds were used as negative controls. ELISA was used to measure the antibody titers of vaccinated birds. The results of protection studies are shown in Table 2. The purified native X-73 OmpH induced 100% protection against homologous strain challenge. The protease-treated native OmpH and recombinant protein induced no protection.

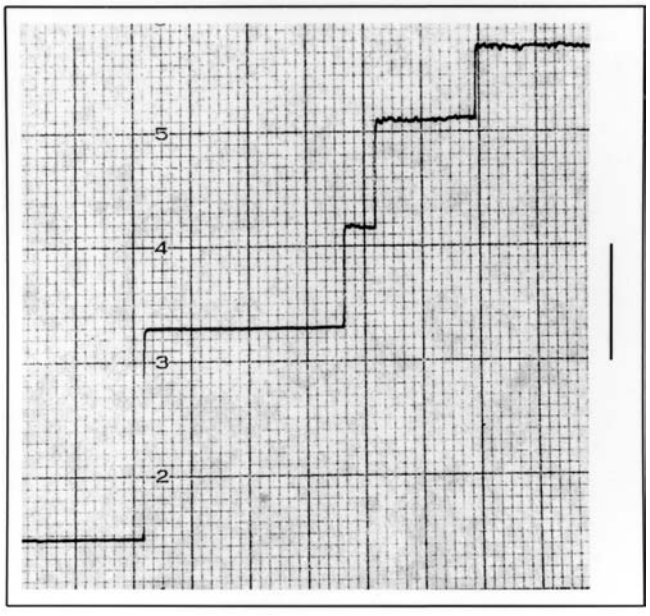

FIG. 6. Chart recording of the stepwise increase in conductance caused by the addition of purified X-73 OmpH to the aqueous phase (1 M KCl), bathing a lipid bilayer membrane made from 1.5% oxidized cholesterol in *n*-decane. The applied voltage was 50 mV. The *x* axis is chart speed, at 5 cm/min. The *y* axis is volts, at 200 mV/unit (units correspond to the vertical bar on the right).

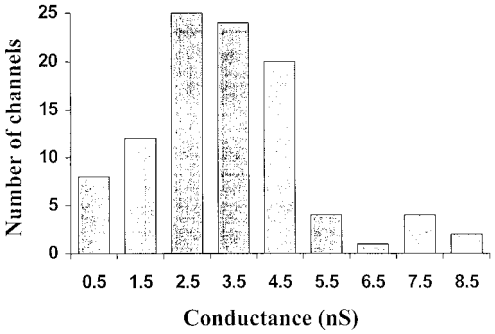

FIG. 7. Histogram of conductance steps in 1.0 M KCl. The membrane was made with 1.5% oxidized cholesterol in *n*-decane over a hole of 0.1 mm^2 separating the two aqueous compartments. Purified X-73 OmpH was added to one compartment and 50 mV was applied. The total number of conductance steps examined was 100. The average single channel conductance was 0.62 nS.

DISCUSSION

The original purpose of this study was to clone and express the in vivo-expressed cross-protection factors, the 179- and 153-kDa outer membrane proteins of serotype 3 (42–44). Because their N-terminal amino acid sequences are almost identical to that of the reported porin H (8), we presumed that these proteins may be different forms (oligomers) of the same monomer.

Since the major outer membrane proteins of several strains of *P. multocida* have been reported to show general properties of other bacterial porins (8, 19, 24), we presumed that *P. multocida* X-73 major outer membrane protein OmpH, with a molecular mass of about 37 kDa, might also be a porin. The method used to purify the major outer membrane porin, porin H of *P. multocida* serotype D2, as described by Chevalier et al. (8), was used in this study for the purification of X-73 major outer membrane protein OmpH. The trimeric or oligomeric form of OmpH was purified by selective extraction of the disrupted bacterial cells with sodium *N*-lauroyl sarcosinate and SDS, followed by size exclusion chromatography. Although the OmpH was significantly purified, the protein still contained a trace amount of LPS detected by the KDO method. Porins usually have a strong association with LPS. It is difficult to obtain a porin completely free of LPS contamination (11, 37). OmpH showed heat-modifiable properties when it was analyzed by PAGE. The fully denatured monomer of OmpH has

TABLE 2. Protection of vaccinated chickens following challenge with *P. multocida* X-73[a]

Antigen	ELISA titer against homologous antigen[b]		No. dead (%)[c]
	Prevaccination	Postvaccination	
X-73 OmpH	10	56,234	0 (0)*
Recombinant protein	10	30,000	9 (82)
X-73 OmpH (protease treated)	ND	ND	10 (91)
X-73 bacterin	10	31,623	0 (0)*
None (control)[d]	ND	ND	9 (82)

[a] Chickens were challenged with 100 CFU of X-73/bird and were vaccinated at 5 and 8 weeks of age.
[b] Antibody titer was measured pre- and postvaccination. Antisera of a group were pooled and measured two times. ND, not determined.
[c] Number of dead chickens out of 11 total. Values followed by asterisks are significantly different from those without asterisks ($P < 0.01$).
[d] Controls were not vaccinated.

a molecular mass of approximately 37 kDa. The unboiled OmpH displayed a ladder of high-molecular-mass proteins, which may represent the trimeric or oligomeric form of OmpH and may also be associated with LPS, as has been described for other putative *P. multocida* porins (19). The N-terminal amino acid sequence of OmpH is very similar to other putative *P. multocida* porins, including porin H. They might be the same major outer membrane porin of *P. multocida*. The minor difference might be because they are isolated from different strains or serotypes.

Bacterial porin genes are sometimes difficult to clone in *E. coli* because foreign porins are usually lethal for *E. coli* (6, 13). In this study, we developed an effective and fast cloning strategy in which the gene was isolated by a combination of genomic library construction and PCR amplification. The *ompH* gene of X-73 was successfully isolated and sequenced. We further used *Pfu* DNA polymerase (Stratagene), which has a proofreading function, to amplify the whole *ompH* gene from genomic X-73 DNA, and we found the sequence of the amplified PCR product to be identical to the initially obtained *ompH* gene sequence (data not shown). The sequence of the *ompH* gene predicted a protein (OmpH) which has characteristics typical of gram-negative bacterial porins. The amino acid sequence shows similarities to other bacterial porins and has the highest similarity to *H. influenzae* protein P2 (38% identity). *Pasteurella* and *Haemophilus* species share strong taxonomic associations, so the high similarity of OmpH to protein P2 is reasonable. Protein P2 has been characterized as a porin (40).

In the expression experiments, we tried unsuccessfully to subclone the whole *ompH* gene, which encodes primary protein containing a signal peptide, in *E. coli*. This failure could be because of the leaking expression of the primary protein when no IPTG was added. We then tried to subclone the primary protein gene into the pRSET expression vector system (Invitrogen, San Diego, Calif.), in which the insert was positioned after the T7 promoter and the expression of the inserted gene occurred only after *E. coli* was infected with M13/T7 helper phage. This was also unsuccessful (data not shown). The reason for this is unknown. It was interesting that although the primary OmpH was lethal for *E. coli*, the mature protein of OmpH was not. The reason for this is unknown. Perhaps this is because the signal peptide in the primary protein helped to target OmpH to the outer membrane of *E. coli*. The integrated OmpH may cause osmotic destabilization of the cells, displacement of *E. coli* porins, or a change in the structural integrity of the outer membrane (6). For mature protein of OmpH, there is no signal peptide and the protein is kept in the cytoplasm. This appeared to produce no disturbance to the outer membrane of *E. coli.*

In library construction and screening, we did not obtain the clone containing the *ompH* gene from the *Sau*3AI genomic library, but we obtained a partial sequence of the *ompH* gene from the *Taq*I genomic library. We now know the reason. In the *ompH* gene sequence, there are two *Sau*3AI restriction sites in the upstream and downstream regions of the gene. There are three *Taq*I restriction sites with two sites in the promoter region and one site in the middle of the coding region of *ompH*. So, in the *Sau*3AI library, even completely digested *P. multocida* genomic DNA insert in the plasmid may contain the whole *ompH* gene expressing the primary OmpH, which is lethal to *E. coli*. But in the *Taq*I library, the insert in the plasmid contained only a partial *ompH* gene, without the promoter. This partial gene did not express or expressed only a partial OmpH protein, which may not be lethal to *E. coli.*

The previously reported high-molecular-mass cross-protection factors (42–44) may be the native trimeric or oligomeric forms of OmpH which were not fully solubilized during Western blotting. The differences in molecular masses of the cross-protection factors at high-molecular-mass positions may be due to the association of different amounts of LPS to the protein (19). Actually, the N-terminal amino acid sequences of the cross-protection factor 179- and 153-kDa proteins (which were from strain P-1059) are completely identical to that of strain P-1059 OmpH (data not shown). However, the relationship between OmpH and the cross-protection factors needs to be studied further.

In the planar lipid bilayer assay, the native X-73 OmpH demonstrated pore-forming activity. This experiment firmly proved that the X-73 major outer membrane protein is a porin. The recombinant OmpH expressed in *E. coli* showed no pore-forming function. This may be because the recombinant OmpH was not properly folded in *E. coli* or because the protein was denatured during purification due to the use of strong denaturants such as guanidine hydrochloride and urea. We tried unsuccessfully to refold the denatured recombinant protein into the native trimer or oligomer conformation. The reason for our inability to do so was unknown. Probably, it was because the fusion of 13 amino acids at the N terminus of OmpH interfered with the refolding process.

In the protection studies, the purified native X-73 OmpH induced 100% protection, as did the X-73 bacterin. But the recombinant OmpH induced little protection. ELISA showed that both proteins stimulated high titers of antibodies against homologous antigens. Western blotting showed that the antibodies induced by the recombinant protein reacted to denatured X-73 OmpH but did not react to undenatured native X-73 OmpH, while the antibodies induced by native OmpH were able to react to undenatured native X-73 OmpH (data not shown). These results indicated that the recombinant OmpH was a denatured protein which could not induce antibodies against native conformational epitopes of X-73 OmpH. We also did an ELISA which showed that the antiserum induced by the recombinant protein contained very low antibody titers against native OmpH in the whole-cell lysate of X-73 (data not shown). According to previous reports of immunization studies with other bacterial porins, the trimeric or native conformation of porin is considered crucial for induction of protective immunity (25, 27, 45). We also examined the induction of cross-protection by X-73 OmpH in turkeys against strain P-1059 (serotype 3) challenge; the results showed that X-73 OmpH induced little and inconsistent cross-protection (data not shown). Although the purified X-73 OmpH still contained a small amount of LPS, the results in Table 2 showed that the induction of homologous protection was due to the protein, not to the contaminated LPS. This was further confirmed by the fact that a synthetic peptide derived from the predicted amino acid sequence of OmpH provided 70% protection in chickens against lethal X-73 challenge (data not shown). It is also unlikely that the protection was induced by the trace amount of contaminated proteins.

The hybridization of the X-73 *ompH* gene sequence with the chromosomal DNAs of the other *P. multocida* serotypes and the reaction of antiserum against X-73 recombinant OmpH with a protein band of the other somatic serotypes indicate that the OmpH gene is conserved among all of the *P. multocida* serotypes.

ACKNOWLEDGMENTS

This work was supported by funds provided by the United States Poultry & Egg Association. Functional assays of the proteins were also supported by financial assistance to Robert E. W. Hancock from the

Medical Research Council of Canada and from an MRC Distinguished Scientist Award.

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