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Immunological Characterization of Full and Truncated Recombinant Clones of *ompH(D:4)* Obtained from *Pasteurella multocida* (D:4) in Korea

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Abstract We cloned a gene of *ompH(D:4)* from pigs infected with *P. multocida* D:4 in Korea [16]. The gene is composed of 1,026 nucleotides coding 342 amino acids (aa) with a signal peptide of 20 aa (GenBank accession number AY603962). In this study, we analyzed the ability of the *ompH(D:4)* to induce protective immunity against a wild-type challenge in mice. To determine appropriate epitope(s) of the gene, one full and three different types of truncated genes of the *ompH(D:4)* were constructed by PCR using pET32a or pRSET B as vectors. They were named *ompH(D:4)-F* (1,026 bp [1-1026] encoding 342 aa), *ompH(D:4)-t1* (693 bp [55-747] encoding 231 aa), *ompH(D:4)-t2* (561 bp [187-747] encoding 187 aa), and *ompH(D:4)-t3* (540 bp [487-1026] encoding 180 aa), respectively. The genes were successfully expressed in *Escherichia coli* BL21(DE3). Their gene products, polypeptides, OmpH(D:4)-F, -t1, -t2, and -t3, were purified individually using nickel-nitrilotriacetic acid (Ni-NTA) affinity column chromatography. Their M_rs were determined to be 54.6, 29, 24, and 23.2 kDa, respectively, using SDS-PAGE. Antisera against the four kinds of polypeptides were generated in mice for protective immunity analyses. Some 50 μg of the four kinds of polypeptides were individually provided intraperitoneally with mice (n=20) as immunogens. The titer of post-immunized antiserum revealed that it grew remarkably compared with pre-antiserum. The lethal dose of the wild-type pathogen was determined at 10 μl of live *P. multocida* D:4 through direct intraperitoneal (IP) injection, into post-immune mice (n=5, three times). Some thirty days later, the lethal dose (10 μl) of live pathogen was challenged into the immunized mouse groups [OmpH(D:4)-F, -t1, -t2, and -t3; n=20 each, two times] as well as positive and negative control groups. As compared within samples, the OmpH(D:4)-F-immunized groups showed lower immune ability than the OmpH(D:4)-t1, -t2, and -t3. The results show that the truncated-OmpH(D:4)-t1, -t2, and -t3 can be used for an effective vaccine candidate against swine atrophic rhinitis caused by pathogenic *P. multocida* (D:4) isolated in Korea.

Key words: *Pasteurella multocida* (D:4), OmpH, immune protection, vaccine candidate, pig atrophic rhinitis

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Pasteurella multocida is a renowned veterinary pathogen that causes economically devastating infectious diseases in swine, fowl, and many other domesticated animals [6, 27]. Respiratory disease, atrophic rhinitis in swine, is known to be caused by type D of *P. multocida* [6, 16, 23]. This suite of diseases has been poorly controlled and still represents a severe problem in the domestic animal industry. Considerable research on the mechanisms of immunity, host preference, pathogenesis, and virulence of *P. multocida* have resulted in only very small increases in our understanding of the organism [9, 19]. Outer membrane protein Hs (OmpHs) of various strains of *P. multocida* are thought to be attractive vaccine candidates to induce homologous and/or heterologous immunities against the infections [7, 9, 17, 18]. The OmpH has a homotrimeric conformation and stability in sodium dodecyl sulfate (SDS) at room temperature. The molecular masses of dissociated monomers vary between 34 and 42 kDa depending upon their serotypes of the bacteria and electrophoretic system used for analysis [7, 16, 17]. To date, neither the precise mechanisms of immunity nor the specific bacterial antigens involved in immunity to pasteurellosis have been clearly identified. Safe and effective vaccines against pasteurellosis are still lacking, and until recently there has been no extensive characterization of this organism at the molecular level [12]. The inactivated and live vaccines that are currently obtained with selected and purified OMP have intrinsic defects, despite their advantages [19]. Whole cell bacterins (inactivated vaccine) can supply some degree of effect in controlling clinical diseases, but only against the homologous LPS serotype. There is good evidence that cross-protective antigens are expressed only under *in vivo* conditions. A major benefit to using live, attenuated vaccines is the fact that they are able to stimulate cross-protection against heterologous serotypes of bacteria. However, because the basis for attenuation is vague, reversion to virulence is not uncommon [11]. Unfortunately, our insufficient understanding of the diseases' pathogenesis and the related lack of effective vaccine development have resulted in a great loss to the domestic animal industry as well as to the world's food supply. In the United States alone, the economic loss due to bovine respiratory diseases has been as much as 640 million dollars per year [3].

Several studies have demonstrated the potential that research in OMPs has for the development of a protective antibody response against Gram-negative bacteria. For example, the D15 protein of *Haemophilus influenzae*, which has 75% aa identity with Oma87 of *P. multocida* [20], has been displayed in passive immunization studies to protect infant rats against *H. influenzae* bacteremia [17]. It has been shown that a 20-kDa N-terminal fragment of the D15 protein contains protective epitopes against *H. influenzae* types a and b [29]; and P6 OMP of *H. influenzae* has been seen to elicit protective immunity in animal models of infection. Furthermore, a gene encoding a homologous protein of P6 in *H. influenzae* has been cloned and identified in all 16 somatic serotypes [13]. Vaccination of rabbits [18], chickens [30], and mice [27] with *P. multocida* OMPs stimulated significant protection against a challenge from live bacteria, and cytokine expression, and it has been reported that high antibody responses to several *P. multocida* OMPs had some correlation with resistance to experimental bovine pneumonic pasteurellosis [8].

In this research, we cloned and expressed a full and three kinds of truncated *ompH(D:4)*; *ompH(D:4)*-t1, -t2, and -t3 from a Korean isolate of *P. multocida* D:4. The truncated genes (*ompH(D:4)*-t1, -t2, and -t3) were obtained from pGEM-T/OmpH using *ompH(D:4)*-specific primers. The immunogenicity and potential roles of truncated proteins (OmpH(D:4)-F, -t1, -t2, and -t3) in inducing protective immunity against *P. multocida* D:4 were analyzed in mice. These results show that the three truncated recombinant clones (*ompH(D:4)*-t1, -t2, and -t3) can be used for an effective vaccine candidate against swine atrophic rhinitis in Korea.

MATERIALS AND METHODS

Reagents, Enzymes, Bacterial Strains, and Culture Conditions

Chemicals and necessary items for this research were obtained from various sources: bacterium culture media (Difco, U.S.A.); enzymes and gene induction reagents (Stratagene, U.S.A.); gel electrophoresis (acrylamide and agarose), and immunization and immunoblotting reagents (Sigma, Bio-Rad, U.S.A.); organic solvents (Merck, Germany); DNA modification enzymes, polymerases, and restriction endonucleases (New England Biolabs, U.S.A.). Other chemicals used were of the highest purity available (Sigma, U.S.A.). *Pasteurella multocida* D:4 (Korean isolate) was grown to sporulation as reported (Aronson *et al.*, 1971). The plasmids were fractionated by sucrose gradients (5–25% sucrose in 0.005 M EDTA, 0.55 M NaCl, 0.05 M Tris-HCl) centrifugation, and they were analyzed by agarose (0.8%) gel electrophoresis. The host for gene cloning was *Escherichia coli* DH5α (*supE44 ΔlacU169 [Φ80lacZΔM15] hsdR17 recA1 endA1 gyrA96 thi-1 relA1*). The strain was cultured in LB medium and was harvested to use for transformation [24]. A pGEM-T easy vector (Promega, U.S.A.) was used as a cloning vector. It was transformed into *E. coli* DH5α [15].

Molecular Cloning of Full and Truncated *ompH(D:4)*s by PCR

Full- and different-lengths of the *ompH(D:4)* were amplified by PCR, and then were cloned in *E. coli* [16]. The entire

Full Open Reading Frame of pGEM-T/OmpH(D:4)

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· · · · · · · · · · · · · GGGATTGTG ATACTATGAAAAAGA · · · ·        21
                                55 F1 →
· · · · · · · · · · · · · · CAGCAA ACGCAGCAACAGTTTACAATCAAGAC      81
                                      F2 →
GGTACAAAAGTTGAT · · · · · · · · · TTAATCCTTAAAAAAGAAAAAGATAAA      141
                                                  187
CGTGGTGATTTAGTGGATAACGGTTCACGCGTTTCTTTCAAAGCAT CTCATGACTTAGGC     201
                                                   F3 →
GAG · · · · · · · · · · · · · CTCCGTTTCAGTACAAAAGAGGAAGTAGAA      261
GTTACACAAAATCAAAAAGTAGTTCGTAAATACAAGGTTGAACGAATTGGTAACGATGTT      321
CATGCAAAACGTCTT · · · · · · · · · · · · · · · · · · · ·           381
· · · · ACTATTGGTGAT · · · · GTGTCTGAC · · · · · · · · · ·        441
                                             487
· · · · · · · · · · GGTGAAAAAGCAATTAACTTCAAGT CTGCAGAATTCAAC      501
                                                  F4 →
GGTTTC · · · · · · · · · · · · · · · · · · · AAACAAGCAGCACGT       561
GACGGTCGCGGT · · · · · · · · · · · · · AAAATGGGTGATGTTGGT · ·     621
· · · · · · · · · AGCCAAGAATATGTAACAGAAACAGCCAAACAAGAAAAAGAA      681
AAAGCCTTTATGGTC · · · · · · · · · · · · · · · · · · GACTAC       741
   747
GCACAA TCTAAAGTGACTAACGTAGATGGTAAAAAACGTGCACTTGAAGTGGGC · · ·    801
  ← R2
· · · CTTAACGATAAAGCGAAAGTTTACACTGAT · · · · · · · AAAGGTCCA     861
AAAGGTGCGACTACAAGAGATCGCGCTATCATC · · · · · · · · · · · · ·      921
CAAGTTGAAACTTTTGTTGAAGGTGGT · · GGCAGAACTAAAAAAGCAGCTGGCGTAACA    981
                                                     1026
ACTAAAGATAACAAAGTTGGCGTTGGTTTACGCGTACACTTCTAA TTTTAGTTAGAATCT    1041
                                              *   ← R1
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Note : Restricotion site at 5'-*Bam*HI(GG/ATCC, →), at 3'-*Hind*III (AA/GCTT, ←)

Fig. 1. Primer annealing regions on pGEM-T/OmpH(D:4).
There are six [4 forward (F1, 2, 3, and 4), 2 reverse (R1, R2)] primers to amply full or truncated DNA fragment of *ompH(D:4)*. Amplification with F1 and R1 generates a full-length ORF. Amplification with F2 and R2 generates truncated ORF-t1. Amplification with F3 and R2 generates truncated ORF-t2. Amplification with F4 and R1 generates truncated ORF-t3.

Table 1. Nucleotide sequences of primers. Forward primers were designed with a BamHI (GG/ATCC) sequence; reverse primers with a HindIII (AA/GCTT) sequence.

Forward (F)/Reverse (R) primer	Nucleotide sequence
F1	5' GGGATTGGGGATCCTATGAAAAAGA 3' (25 mer)
F2	5' CAGCGGATCCAGCAACAGTTTACAA 3' (25 mer)
F3	5' AAAGCGGATCCTGACTTAGGCGAG 3' (24 mer)
F4	5' TTCAAGGATCCAGAATTCAACGGT 3' (24 mer)
R1	5' CTAACAAAGCTTAGAAGTGTACGCG 3' (25 mer)
R2	5' AGTCACTTAAGCTTGTGCGTAGTC 3' (24 mer)

open reading frame (ORF) of *ompH(D:4)*, named pGEM-T/OmpH(D:4) [15, 16], was used for the PCR template. Forward (F-1, -2, -3, and -4) and reverse (R-1 for F-1 and -4 and R-2 for F-2 and -3) primers were designed for the PCR to amplify entire or truncated ORFs of the gene; *ompH(D:4)*-F (F-1 and R1), -t1 (F-2 and R-2), -t2 (F-3 and R-2), and -t3 (F-4 and R-1). The forward primers have a BamHI site, and the reverse primers have the HindIII site. Their names and nucleotide sequences along with annealing positions to the template are shown in Fig. 1 and Table 1.

Amplification of the ORF was performed as before [16]. The PCR-amplified *ompH(D:4)*-F, -t1, -t2, and -t3 DNAs were digested with BamHI and HindIII to produce sticky-ended fragments. Vectors pET32a and pRSET B were digested with BamHI and HindIII. The *ompH(D:4)*-F was ligated with pET32a, whereas *ompH(D:4)*-t1, -t2, and -t3 were ligated with pRSET B vectors, respectively. The recombinant phagemids were transfected into the host cells of *E. coli* DH5α. Transformants were selected by α-complementation on LB-ampicillin (100 mg/ml) agar plates. The clones were named pET32a/OmpH-F, pRSET B/OmpH-t1, pRSET B/OmpH-t2, and pRSET B/OmpH-t3, respectively. The nucleotide compositions of these recombinant clones were determined by the method of Sanger *et al.* [25].

Expression and Purification of Full and Truncated OmpH(D:4)s & Identification of the Proteins by SDS-PAGE

Four recombinant clones were expressed in *E. coli* BL21(DE3) [(F^- *ompT* $hadS_B$ ($r_B^-m_B^-$) *gal dcm* (DE3)]. Recombinant proteins were purified as previously described [16]. Protein concentration was determined by the method of Bradford [4].

Polyacrylamide gel (12.5%) electrophoresis was performed under denaturing conditions with SDS (0.1%) using a vertical slab gel unit with 1.5-mm spacer arms (Hoeffer Scientific, U.S.A.) [14]. The desired quantity of protein was loaded into a well and supplied with a constant power (5 volts/cm). SDS-polyacrylamide gels were stained with Coomassie Brilliant Blue.

Vaccination and Production of Anti-OmpH(D:4)s (-F, -t1, -t2, and -t3) Antisera and Enzyme-Linked Immunosorbent Assay

Immunization procedures were basically the same as Ryou and Kwon [24] with a minor modification. Purified OmpH(D:4)-F, -t1, -t2, and -t3 (50 μg/mouse) as immunogens were mixed with an equal volume of Freund's Complete Adjuvant (FCA). The mixtures were intraperitoneally (IP) injected to BALB/c mice (6–8-week-old, ~20 g/head). The immunization was performed three times with a 10-day interval. Freund's Incomplete Adjuvant (FIA) was substituted for the FCA from the second immunization. Some 200 μl of blood was collected from the eye vein of the mouse one week after each immunization, and antiserum was isolated from the whole blood by centrifugation. Mice were challenged IP with virulent wild *P. multocida* D:4 ten days after the third immunization. Then, the survival rate of each group of mice was recorded 72 h later.

Enzyme-linked immunosorbent assays (ELISA) for the detection of anti-OmpH(D:4)s (-F, -t1, -t2, and -t3) antisera were performed on flat-bottomed 96-well plates (Costa, U.S.A.) coated with 0.1 μg/well of purified OmpH in 0.05 M carbonate buffer (pH 9.6) at 37°C for 1 h. The rest of the procedures for the analyses were the same as the manufacturer's directions.

RESULTS

Cloning a Full and Three Truncated *ompH(D:4)*s from *P. multocida* D:4 (Korean Isolate)

To investigate plausible antigenic determinant(s) of the gene, *ompH(D:4)*, a full and three truncated recombinant DNA fragments of the gene had been cloned using pET32a/OmpH-F as template (Fig. 2A, Lanes 2–4). The composition of the gene and the primer-annealing sites are presented in Fig. 1. The nucleotide sequences of the forward and the reverse primers appeared in Table 1. The full and three recombinant gene fragments amplified by PCR were resolved on agarose gel to be 1,026 bp-, 693 bp-, 561 bp-, and 540 bp-long, respectively (Fig. 2A, lanes 1–4). The 1,026-bp-long DNA fragment was ligated with pET32a vector

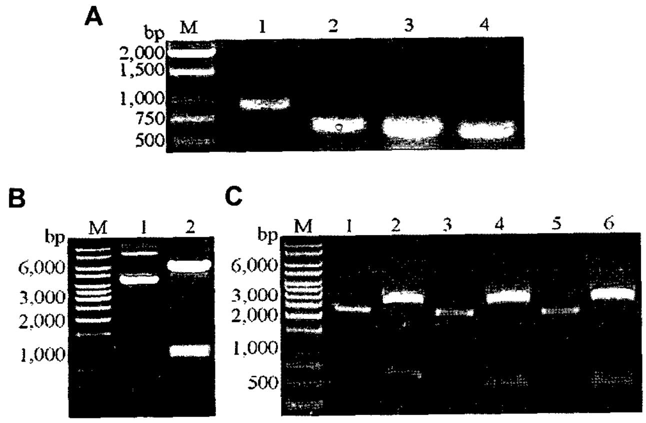


Fig. 2. Construction of four recombinant clones, *ompH(D:4)s*-F, -t1, -t2, and -t3.
A. DNA fragments amplified by PCR. Lane M, 1-kb DNA ladder marker; lane 1, full length of *ompH(D:4)-F* (1,026 bp); lane 2, truncated *ompH(D:4)-t1* (693 bp); lane 3, truncated *ompH(D:4)-t2* (561 bp); lane 4, truncated *ompH(D:4)-t3* (540 bp). B. Recombinant clone, *ompH(D:4)s-F*. Lane M, 1-kb DNA ladder marker; lane 1, full length of pET32a/OmpH plasmid; lane 2, BamHI and HindIII enzyme digestion of pET32a/OmpH (1,026 bp). C. Lane M, 1-kb DNA ladder marker; lane 1, pRSET B/OmpH-t1 plasmid; lane 2, BamHI and HindIII enzyme digestion of pRSET B/OmpH-t1 (693 bp); lane 3 pRSET B/OmpH-t2 plasmid; lane 4, BamHI and HindIII enzyme digestion of pRSET B/OmpH-t2 (561 bp); lane 5, pRSET B/OmpH-t3 plasmid; lane 6, BamHI and HindIII enzyme digestion of pRSET B/OmpH-t3 (540 bp).

Fig. 3. SDS-PAGE profile of recombinant fusion polypeptides. The samples were loaded on polyacrylamide gel (15%).
Lane M, protein molecular weight standard marker; lane 1, OmpH(D:4)-F, 54.6 kDa; lane 2, OmpH(D:4)-t1, 29 kDa; lane 3, OmpH(D:4)-t2, 24 kDa; lane 4, OmpH(D:4)-t3, 23.2 kDa.

restricted by BamHI-HindIII to generate a recombinant plasmid, pET32a/OmpH, named *ompH(D:4)*-F (Fig. 2B). It contained the entire ORF of *ompH(D:4)* encoding 342 aa residues with a signal peptide (20 aa). The other three DNA fragments (693 bp-, 561 bp-, and 540 bp-long) were ligated with BamHI-HindIII-cut pRSET B vectors, respectively, to produce recombinant clones of pRSET B/OmpH-t1, -t2, -t3, in that order (Fig. 2C). The truncated 693-bp-long *ompH(D:4)-t1* did not contain the signal sequences at the 5' region (from the 1st to the 54th bp), nor some 279 bp at the 3' region (from the 745th to the 1,026th bp) (Fig. 1). In other words, *ompH(D:4)-t1* could not produce signal peptide (hatched-bar in Fig. 4), nor some 93 aa at the C-terminus (Fig. 4). The *ompH(D:4)-t2* (561-bp-long) was 132 bp (from the 55th to the 186th bp) shorter than *ompH(D:4)-t1*. It meant that *ompH(D:4)-t2* did not contain the DNA fragment encoding the first three hydrophilic domains (44 aa) of OmpH(D:4) in comparison with *ompH(D:4)-t1* (Fig. 4). The *ompH(D:4)-t3* had been designed to delete *ca.* half of the gene from the 5'-region (the 1st to the 486th bp) to contain 540 bp from the 3'-end of the gene. Thus, *ompH(D:4)-t3* was able to translate 180 aa from the C-terminus of OmpH(D:4). The goodness of the recombinant plasmids, pRSET B/OmpH-t1, -t2, -t3, had been verified by standard procedures of gene cloning; transfection, colony formation, extraction/purification of amplified plasmids, and restrictions of the ligation sites (BamHI and HindIII) on the DNAs shown in Figs. 2B and 2C. All the four recombinant clones were proven to have right-size inserts with correct nucleotide compositions. Their nucleotide sequences had been confirmed by Sanger *et al.* [25]. They were used to produce antisera against OmpH(D:4)s, for the analyses of the recombinant clones as vaccine to protect atrophic rhinitis in pigs in Korea.

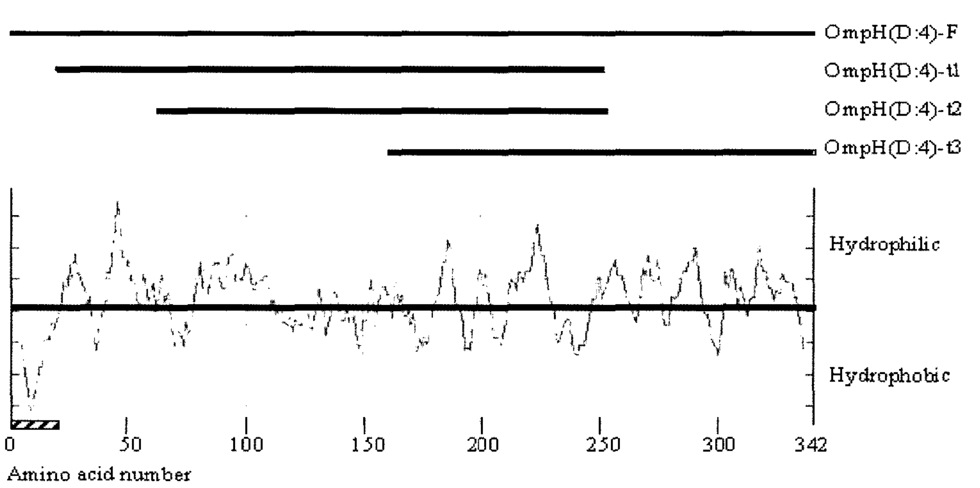
Expression and Purification of different sizes of OmpH(D:4)s expressed by recombinant clones in *E. coli*

All the four recombinant clones generated in this research were successfully expressed in *E. coli* BL21(DE3). As the OmpH(D:4)-F is known to be toxic to the host *E. coli* BL21(DE3), the full ORF was cloned into a fusion vector, pET32a, to reduce instability of the gene expression. The other three truncated DNA fragments were cloned into pRSET B vectors. The gene products named OmpH(D:4)-F, -t1, -t2, and -t3 were purified by the procedure previously described [16]. The M_rs of OmpH(D:4)-F, -t1, -t2, and -t3 were resolved by SDS-PAGE as 54.6, 29, 24, and 23.2 kDa, respectively (Fig. 3, Lanes 1–4). Their concentrations were determined by Bradford assay [4].

Protection Studies

Balb/c mice (10 mice per group) were IP immunized with 50 μg of the purified protein [OmpH(D:4)-F, -t1, t-2, or t-3] three times with a 10-day interval (Fig. 5). As a positive and a negative control, the mice were injected with commercial vaccine and PBS, respectively. Immunizations with the recombinant OmpH(D:4)s and commercial vaccine resulted in higher antiserum responses against the recombinant OmpH(D:4)s than that of the negative control. The strength of antisera against the immunogens are 1.000 in OmpH(D:4)-F; 0.981 in OmpH(D:4)-t1; 0.988 in OmpH(D:4)-t2; 1.047

A



B

*MKKTIVALA VAA VAA TSA*NAATVYNQDGTKVDVNGSVRLILKKEKDKRGDLVDNGSRVS
FKASHDLGEGLSALAYAELRFSTKEEVEVTQNQKVVRKYKVERIGNDVHAKRLYAGFAYE
GLGTLTFGNQLTIGDDVGVSDYTYFLGGINNLLSSGEKAINFKSAEFNGFTFGGAYVFSAG
ADKQAARDGRGFVVAGLYNRKMGDVGLALEAGYSQEYVTETAKQEKEKAFMVGTELSYA
GLALGVDYAQSKVTNVDGKKRALEVGLNYDLNDKAKVYTDLIWAKKGPKGATTRDRAIIL
GAGYKLHKQVETFVEGGWGRTKKAAGVTTKDNKVGVGLRVHF*

Fig. 4. Hydropathy of OmpH(D:4)-F generated by Kyte & Doolittle at ProtScale.
A. The differences among OmpH(D:4)-F, -t1, -t2, and -t3 can be recognized with ease. OmpH(D:4)-t1 does not contain hydrophilic domains at the C-terminus region (19-249 aa), whereas OmpH(D:4)-t2 lacks one hydrophilic domain at the N-terminus. OmpH(D:4)-t3 contains most of the hydrophilic domains at the C-terminus except numbers of hydrophilic domains at the N-terminus (162-342 aa). NOTE: The hatched bar represents the signal peptide by the N-terminus. **B.** Underlined amino acid sequences represent hydrophilic domains, and unmarked amino acid sequences, hydrophobic domains.

in OmpH(D:4)-t3; 0.781 in commercial vaccine; 0.393 in PBS (Table 3).

To determine the lethal dose (100%) for a wild mouse, five different dosages of live *P. multocida* D:4 Korean isolate were injected. The volumes of wild-type pathogen were 200 μl, 100 μl, 50 μl, 25 μl, and 10 μl, respectively. Table 2 shows that the safe dosage of the wild pathogen turned out to be 10 μl (4.04×10^5), since the survival rate of mice in Cage V was the greatest among the five (Table 2). After challenge with virulent *P. multocida* D:4, the mouse administrated by OmpH(D:4)-F, -t1, -t2, and -t3 lived longer than the control in PBS. For example, only one head survived in the negative control group, whereas at least seven heads survived in the positive group ($p<0.05$). Comparatively, the survival rate of mice immunized with OmpH(D:4)-t1, -t2, or -t3 was somewhat greater than that of those immunized with OmpH(D:4)-F containing signal peptide (Table 4).

DISCUSSION

It has been known for a long time that OmpHs in various strains of the pathogenic *P. multocida* have been considered as relatively good candidates for immunogens against pasteurellosis. Several OmpHs were identified in some

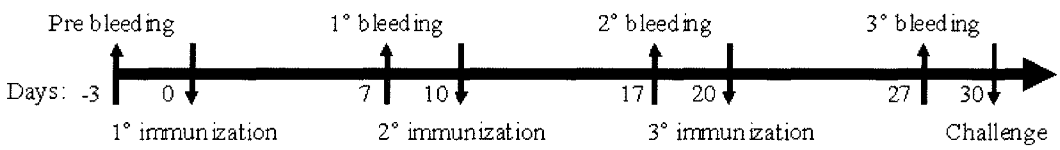


Fig. 5. Time table for immunization and challenge. Mice were intraperitoneally vaccinated three times (days of 0, 10th, and 20th) with four kinds of purified recombinant fusion polypeptides [OmpH(D:4)-F, -t1, -t2, and -t3], commercial vaccine, and PBS, respectively.
At one week after all immunizations, each mouse was bled from the eyes. On the tenth day after the third immunization, the mice were challenged with virulent *P. multocida* (LD_{100}) and monitored for 72 h.

Table 2. Determination of lethal dosage of mouse against live *P. multocida* D:4.

Case	Absorbance (A_{600})	CFU/ml	Mortality (%)			
			Dose (μl)	Day 0	Day 1	Day 2
I	1.7	4.04×10^2	200	100	100	0
II	1.7	4.04×10^2	100	100	100	0
III	1.7	4.04×10^2	50	100	100	0
IV	1.7	4.04×10^2	25	100	100	0
V	1.7	4.04×10^2	10	100	100	20

Table 3. Strength of anti-OmpH(D:4)-F, -t1, -t2, and -t3 antisera by enzyme linked immunosorbent assay (ELISA).

Immunogen	Preimmune serum	Post antiserum (the 3rd-bled)
Recombinant OmpH-F	0.373±0.086	1.002±0.032
Recombinant OmpH-t1	0.403±0.186	0.981±0.162
Recombinant OmpH-t2	0.437±0.147	0.988±0.095
Recombinant OmpH-t3	0.424±0.142	1.04 ±0.047
Commercial vaccine	0.253±0.145	0.781±0.086
PBS	0.215±0.134	0.392±0.241

strains of *P. multocida* to be used in protective experiments against the virulent agents, and suggested that serotypes could be the etiologic agents of the disease [1, 2, 3, 5, 9, 10, 12, 28, 30]. Our results obtained from current studies also agree with the suggestion.

We constructed several recombinant clones of *ompH(D:4)*s to produce full and truncated recombinant fusion polypeptides [OmpH(D:4)-F, -t1, -t2, and -t3] of the outer membrane protein H (D:4). It had been, however, realized that OmpH(D:4)-F had a deleterious effect on the growth of host cells of *E. coli*, since the *ompH (D:4)-F* transformants could not produce any colonies at all on the LB plate [16]. This could be due to the action of the signal peptide of OmpH(D:4)-F. To avoid the harmful activity of the signal peptide, the full gene for the expression of OmpH(D:4)-F had been cloned into pET32a (not pGEM-T, nor pRSET B) to produce fusion-polypeptide in the host. It is desirable to employ this kind of fusion vector to clone the full ORF of a gene, *ompH*. The other three truncated clones [*ompH(D:4)*-t1, -t2, and -t3] were designed to eliminate the DNA fragment (54 bp) coded for the signal peptide (18 aa) to ligate into pRSET B vectors. The two gene expression plasmids were designed to produce some additional peptides besides a cloned gene to generate an appropriate size of fusion protein. The additional aa residues of pET32a are 160 aa including the thioredoxin sequence prior to multiple cloning sites; pRSET B, 31 aa. All the four recombinant clones generated here were stably expressed in the host *E. coli* BL21(DE3) from generation to generation. Furthermore, the purification of the fusion proteins ·is successful using Ni-NTA affinity column chromatography. Their M_rs were estimated to be 54.6 kDa [OmpH(D:4)-F], 29 kDa [OmpH(D:4)-t1], 24 kDa [OmpH(D:4)-t2], and 23.2 kDa [OmpH(D:4)-t3], respectively, by SDS-PAGE. It should be mentioned that these four kinds of fusion polypeptides were administered to mice for our immunological experiments.

Some 20 mice were immunized against each recombinant fusion polypeptide for a one-month period with ten-day intervals. The mice were tamed for three days before the initial immunization. ELISA data (Table 3) indicate that high-strength anti-OmpH(D:4)-F, -t1, -t2, and -t3 antisera have been raised in the four groups of mice. Their titers were >3 times higher than that of the negative control (PBS). However, the protection capabilities of the four recombinant fusion polypeptides against the live pathogen [*P. multocida (D:4)*], in the vaccinated mice, were not equally alike, as appeared in Table 4. It should be mentioned that prior to the challenge, the lethal dosage of the live pathogen was determined to be some 10 μl/head based on physical conditions of the mice before death (cf. Case V in Table 2). Vaccination with the full or the other three truncated recombinant fusion polypeptides [OmpH(D:4)-F, -t1, -t2, and -t3, respectively] rent better protection capability to the mice than those with commercial vaccines (CV). As expected, the protective immunity of PBS was almost none (Table 4). After challenge with the virulent *P. multocida (D:4)*, all of the immunized mice lived longer than non-immunized mice. In other words, the death ratio

Table 4. Protection conferred to immunized mice against live *P. multocida* (D:4).

Immunogen	Dose (μg)	LD_{100} of pathogen (CFU/ml)	Mortality (no. of mouse: mean of duplicate)	Protection(%)
Recombinant OmpH-F	50	4.04×10^2	9/20	45[a]
Recombinant OmpH-t1	50	4.04×10^2	10/20	50[b]
Recombinant OmpH-t2	50	4.04×10^2	10/20	50[b]
Recombinant OmpH-t3	50	4.04×10^2	10/20	50[b]
Commercial vaccine	50	4.04×10^2	7/20	35
PBS	0	4.04×10^2	1/20	5*

[a]p=0.015 compared with *
[b]p=0.028 compared with *

of the immunized mice was much lower than that of non-immunized mice. Thus, it is safe to mention that full- or truncated-OmpH(D:4)-immunized mice have higher immunity against live virulent *P. multocida (D:4)* than immune-free mice. From the challenge data, however, the mortalities of the mice vaccinated with the three truncated fusion polypeptides [OmpH(D:4)-t1, -t2, and -t3] were slightly higher than that of non-truncated, full-size fusion polypeptide [OmpH(D:4)-F]. The difference in figures is not large, however; it might be due to the topological characteristics of the recombinant fusion polypeptides, especially their antigenic determinant(s). To estimate their topologies, a plausible hydropathy of the recombinant full- as well as three truncated-fusion polypeptides has been plotted using the ProtScale program (http://au.expasy.org/tools/protscale.html). The OmpH(D:4)-t1 (18^{th}–249^{th}) is 44 aa residues (spanning two hydrophilic regions) longer than OmpH(D:4)-t2 (62^{nd}–249^{th}) (Fig. 2). However, these two constructs do not contain 93 aa residues from the C-terminus. Since the protective capabilities of these two constructs (-t1 and -t2) are alike, it can be hypothesized that amino acid residues spanning from the 62^{nd} to the 249^{th} could be good enough to elicit the mouse immune system against the virulent pathogen. The vaccination of the mouse with OmpH(D:4)-t3 gave us an interesting result. This truncated fusion polypeptide has been designed to delete some 162 aa residues from the N-terminus leaving rest of 180 aa residues from the C-terminus. However, the efficacy of vaccination with the OmpH(D:4)-t3 is the same as the value with -t1 or -t2 (Table 4). In comparison with the aa composition of -t1 or -t2, the -t3 alone contains some 93 aa residues (250^{th}–342^{th}) from the C-terminus. In other words, the -t3 is the only truncated fusion polypeptide being constructed with the entire C-terminus. Several hydrophilic domains are also found in the 93 aa residues to be considered as important immunogenic determinant(s). A number of previous reports strongly support our results: a truncated polypeptide can induce protective efficacy as well as full-length (size) polypeptide as immunogen in many animal systems [2, 5, 21, 22]. In our experiments, the protective efficacy of the OmpH(D:4)-F is somewhat lower that that of any truncated one. It may be due to the deleterious effect of the signal peptide in eliciting immunity against the pathogen, since only OmpH(D:4)-F has the signal peptide. Our findings indicate that the recombinant full or truncated fusion polypeptides generated in this research can be used as immunogen(s) to challenge against infection of *P. multocida (D:4)* isolated in Korea.

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REFERENCES

1. Adler, B., D. Bulach, J. Chung, S. Doughty, M. Hunt, K. Rajakumar, M. Serrano, A. Zanden, Y. Zhang, and C. Ruffolo. 1999. Candidate vaccine antigens and genes in *Pasteurella multocida*. *J. Biotech.* **73:** 83–90.
2. Baldwin, M. R., J. H. Lakey, and A. J. Lax. 2004. Identification and characterization of the *Pasteurella multocida* toxin translocation domain. *Mol. Microbiol.* **54:** 239–250.
3. Bowland, S. L. and P. E. Shewen. 2000. Bovine respiratory disease: Commercial vaccines currently available in Canada. *Can. Vet. J.* **41:** 33–48.
4. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
5. Busch, C., J. Orth, N. Djouder, and K. Aktories. 2001. Biological activity of a C-terminal fragment of *Pasteurella multocida* toxin. *Infect. Immun.* **69:** 3628–3634.
6. Carter, G. R. 1967. *Pasteurella multocida* and *Pasteurella haemolytica*. *Adv. Vet. Sci. Comp. Med.* **11:** 321–379.
7. Chevalier, G., H. Duchlohier, D. Thomas, E. Shechter, and H. Roblewski. 1993. Purification and characterization of protein H, the major porin of *Pasteurella multocida*. *J. Bacteriol.* **175:** 266–276.
8. Confer, A. W., S. H. Nutt, S. M. Dabo, R. J. Panciera, and G. L. Murphy. 1996. Antibody responses of cattle to outer membrane proteins of *Pasteurella multocida* A:3. *Am. J. Vet. Res.* **57:** 1453–1457.
9. Dabo, S. M., A. W. Confer, and R. A. Quijano-Blas. 2003. Molecular and immunological characterization of *Pasteurella multocida* serotype A:3 OmpA: Evidence of its role in *P. multocida* interaction with extracellular matrix molecules. *Microb. Pathogen.* **35:** 147–157.
10. Dabo, S. M., A. W. Confer, and G. L. Murphy. 1997. Outer membrane proteins of bovine *Pasteurella multocida* serogroup A isolates. *Vet. Microbiol.* **54:** 167–183.
11. Hofacre, C. 1986. A serotypic survey of *Pasteurella multocida* isolated from poultry. *Avian Dis.* **30:** 632–633.
12. Hunt, M. L., B. Adler, and K. M. Townsend. 2000. The molecular biology of *Pasteurella multocida*. *Vet. Microbiol.* **72:** 3–25.
13. Kasten, R. W., L. M. Hansen, J. Hinojoza, D. Bieber, W. W. Ruehl, and D. C. Hirsh. 1995. *Pasteurella multocida* produces a protein with homology to the P6 outer membrane protein of *Haemophilus influenzae*. *Infect. Immun.* **63:** 989–993.
14. Kim, T. W., H. S. Song, and H. Y. Kim. 2005. Distribution of dominant bifidobacteria in the intestinal microflora of Korean adults and seniors, identified by SDS-PAGE of whole cell proteins and 16S rDNA sequence analysis. *J. Microbiol. Biotechnol.* **15:** 388–394.
15. Kim, S.-Y. and J.-Y. Cho. 2005. A modified PCR-directed gene replacements method using λ-red recombination functions in *Escherichia coli*. *J. Microbiol. Biotechnol.* **15:** 1346–1352.
16. Lee, J., S. Kang, S. I. Park, H.-J. Woo, and M. Kwon. 2004. Molecular cloning and characterization of the gene for outer

membrane protein H in a *Pasteurella multocida* (D:4) isolate from pigs with atrophic rhinitis symptoms in Korea. *J. Microbiol. Biotechnol.* **14:** 1343–1349.

17. Loosmore, S. M., Y. P. Yang, D. C. Coleman, J. M. Shortreed, D. M. England, and M. H. Klein. 1997. Outer membrane protein D15 is conserved among *Haemophilus influenzae* species and may represent a universal protective antigen against invasive disease. *Infect. Immun.* **65:** 3701–3707.
18. Lugtenberg, B., R. Boxtel, D. Evenberg, M. Jong, P. Storm, and J. Frik. 1986. Biochemical and immunological characterization of cell surface proteins of *Pasteurella multocida* strains causing atrophic rhinitis in swine. *Infect. Immun.* **52:** 175–182.
19. Luo, Y., J. R. Glisson, M. W. Jackwood, R. E. Hancock, M. Bains, I. H. Cheng, and C. Wang. 1997. Cloning and characterization of the major outer membrane protein gene (ompH) of *Pasteurella multocida* X-73. *J. Bacteriol.* **179:** 7856–7864.
20. Mitchison, M., L. Wei, J. Kwang, I. Wilkie, and B. Adler. 2000. Overexpression and immunogenicity of the Oma87 outer membrane protein of *Pasteurella multocida*. *Vet. Microbiol.* **72:** 91–96.
21. Orth, J. H., D. Blocker, and K. Aktories. 2003. His1205 and His1223 are essential for the activity of the mitogenic *Pasteurella multocida*. *J. Comp. Pathol.* **94:** 203–214.
22. Pullinger, G. D., R. Sowdhamini, and A. J. Lax. 2001. Localization of functional domains of the mitogenic toxin of *Pasteurella multocida*. *Infect. Immun.* **69:** 7839–7850
23. Rutter, J. M. 1985. Atrophic rhinitis in swine. *Adv. Vet. Sci. Comp. Med.* **29:** 239–279.
24. Ryou, C. and M. Kwon. 2000. Immunochemical studies on expression of quinoproteins in *Escherichia coli*. *J. Microbiol. Biotechnol.* **10:** 95–98.
25. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74:** 5463–5467.
26. Shin, H.-J., S.-K. Lee, J. J. Choi, S. Koh, J.-H. Lee, S.-J. Kim, and S.-T. Kwon. 2005. Cloning, expression, and characterization of a family B-type DNA polymerase from the hyperthermophilic crenarchaeon *Pyrobaculum arsenaticum* and its application to PCR. *J. Microbiol. Biotechnol.* **15:** 1359–1367.
27. Vasfi, M., J. Harel, and K. R. Mittal. 1997. Identification by monoclonal antibodies of serotype D strains of *Pasteurella multocida* representing various geographic origins and host species. *J. Med. Microbiol.* **46:** 1–8.
28. Wang, C. and J. R. Glisson. 1994. Identification of common antigens of serotype 1 and serotype 3 *Pasteurella multocida* in poultry expressed *in vivo*. *Avian Dis.* **38:** 334–340.
29. Yang, Y., W. R. Thomas, P. Chong, S. M. Loosmore, and M. H. Klein. 1998. A 20-kilodalton N-terminal fragment of the D15 protein contains a protective epitope(s) against *Haemophilus influenzae* type a and type b. *Infect. Immun.* **66:** 3349–3354.
30. Zhang, H., A. J. Ainsworth, and R. D. Montgomery. 1994. Use of a 35.5 kDa cell membrane composition of *Pasteurella multocida* and an anti-idiotype antibody to induce protective immunity in leghorn chickens. *Vet. Immunol. Immunopathol.* **41:** 89–100.