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Genetic Variations of Outer Membrane Protein Genes of *Vibrio harveyi* Isolated in Korea and Immunogenicity of OmpW in Olive Flounder, *Paralichthys olivaceus*

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한국에서 분리된 *Vibrio harveyi* 외막단백질의 유전적 차이와 넙치*(Paralichthys olivaceus)*에 대한 OmpW의 면역원성 분석

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Abstract

Vibrio harveyi is a pathogenic marine bacterium causing systemic symptoms resulting in mass mortalities in fishes and shrimps in aquaculture. Outer membrane proteins(OMPs) are related to the pathogenicity and thus good targets for diagnosis and vaccination for Gram negative bacteria. Recently vaccination strategies using the OMPs have been suggested to control vibriosis in several fish species. In this study, we have isolated *V. harveyi* from diseased marine fishes from different regions of Korea and investigated genetic variations of four OMP genes including OmpK, OmpU, OmpV and OmpW. Consequently, OmpK and U genes could be divided into 3 subgroups of type I, II, III and type A, B, C, respectively, without any correlation with geographical regions and species while OmpV and W were highly homologous. OmpW gene of *V. harveyi* FP4138 was fully sequenced and predicted the deduced amino acid sequence to form β-barrel with hydrophobic channel. Indeed, the immunogenicity of recombinant OmpW produced in *Escherichia coli* was assessed by vaccinating flounder. As a result, the high antibody response with antibody titer of 4.2±0.7 and protection with relative percent survival of 60% against artificial infection of *V. harveyi* were demonstrated. This result indicates that OmpW is a virulence related factor and it can be a vaccine candidate to prevent a high mortality caused by *V. harveyi* infection in olive flounder, *Paralichthys olivaceus*.

Key words : *Paralichythys olivaceus*, *Vibrio harveyi,* Outer membrane protein, Genetic variation, Immungenicity

Ⅰ. Introduction

In South Korea, olive flounder(*Paralichthys olivaceus*) is the most commercially important fish species as its production(43,413ton in 2014, www.kosis.kr) took up to 50% of the total production of marine fish aquaculture . However, the frequent outbreaks of bacterial diseases and the

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outcome of bacterial resistance against antibiotics have been threatening olive flounder aquaculture industry. Main bacterial diseases occurring in marine fishes including olive flounder are edwardsiellosis by *Edwardsiella tarda*, streptococcosis by *Streptococcus iniae, S. parauberis*, and *Lactococcus garvieae* and vibriosis (Kim et al., 2006). Vibriosis has been reported to be caused by *Vibrio harveyi, V. ichthyoenteri, Photobacterium damselae,* and *V. anguillarum* in Korea (Sunaryanto et al., 1986; Kim et al., 2004; Won et al., 2006). *V. ichthyoenteri* is reported as a pathogen of bacterial white enteritis occurring to olive flounder(Fukuda et al., 1996). *V. anguillarum* and *P. damselae* are very closely related but classified as separated genera from the genus Vibrio(Edward, 1996).

Among the bacteria causing vibriosis, *V. harveyi* is a Gram-negative, motile, and rod shaped bacterium ubiquitous in marine, leading to a systemic disease in marine fishes. Main symptoms caused by *V. harveyi* are skin ulcer and eye lesion, resulting moderate and acute mortalities in fin fishes and invertebrates(Austin and Zhang, 2006). It causes a high mortality of cultured olive flounder and crustaceans such as shrimp (Lavilla-pitogo et al., 1990; Muroga et al., 1990).

Vibriosis caused by *V. harveyi* has been controlled by antibiotics and vaccination. Conventional vaccinations have used formalin killed cells(FKC) and succeeded to achieve good protection and humoral immunity against *V. harveyi* infection. Recent studies have emphasized the role of the outer membrane proteins(OMPs) of pathogenic bacterium in protective immunity for vaccination to prevent vibriosis(Ningqiu et al., 2008; Mao et al., 2007; Qian et al., 2008; Yu et al., 2013). OMPs are channels for ions and nutrients, and embedded in outer membrane, a physical barrier, of Gram negative bacteria. The outer membrane of Gram negative pathogenic bacteria has an important role in the interaction with hosts in the bacterial pathogenicity during adherence, uptake of nutrients from the host, and eliminating host-defense mechanisms (Ellis and Kuehn, 2010).

Vaccination using OMPs in fish seems to be successful as OmpK of *V. harveyi* in orange-spotted grouper(*Epinephelus coioides*)(Ningqiu et al., 2008) and OmpW, V, U, K, TolC of *V. parahaemolyticus* in large yellow croaker(*Pseudosciaena crocea*)(Mao et al., 2007) have been reported to induce good humoral immunity and protection against the pathogens. OmpK was also reported as an effective vaccine candidate for *V. alginolyticus*.(Qian et al., 2008). More recently, Yu et al.(2013) have identified 13 OMPs from a pathogenic *V. harveyi* strain T4D and produced recombinant proteins to test their potentials as a vaccine, demonstrating that Omp173 and Omp214 could induce protective immunity as well as production of specific antibodies. Yu et al.(2013) reported that Omp214 shares 92% homology with OmpW(GenBank: EEZ89828.1) of *V. harveyi*–like 1DA3 isolated from diseased coral(Alves et al., 2010). But since they did not report the sequence of Omp214 it is hard to know the OmpW of Korean isolates of *V. harveyi* would be as effective as Omp214. Indeed Yu et al.(2013) have tested the efficacy of OmpP214 on flounder sized at about 14g which is a smaller size than the common vaccination size for intraperitoneal injection in Korean aquaculture farms.

Moreover, previous studies have neglected the variation of OMPs in different strains. It has been known that genetic variations in OMP genes can

cause different pathogenicity. For example, OmpA gene of 261 *Yersinia enterocolitica* strains could be divided into 23 sequence types and pathogenic group formed five types of Pattern A-E, revealing that most pathogenic strains(155 strains, 91.2% of all of the pathogenic strains) were clustered into Pattern A(Li et al., 2014). Thus, once proved as conserved antigenic proteins of the pathogen, OMPs can be used as subunit vaccines to achieve a cross protection over various serotypes.

In this study, we have isolated *V. harveyi* from diseased marine fishes, which are cultured in different regions of Korea. The genetic variations of four OMP genes including OmpK, OmpU, OmpV and OmpW in the isolated *V. harveyi* were analyzed. In addition, OmpW gene was cloned and performed protein structure homology modeling using the translated amino acid sequences. To elucidate the immunogenicity of OMPs, we have prepared recombinant OmpW in *Escherichia coli* and vaccinated olive flounder. The antibody response and relative percent survival(RPS) were investigated after immunization and challenge test, in relation to future vaccine development.

Ⅱ. Materials and methods

1. Isolation of *V. harveyi* from fish samples

Vibrio was isolated from diseased marine fishes including olive flounder(*Paralichthys olivaceus*), red sea bream(*Pagrus major*), black rock fish(*Sebastes schlegelii*) and flathead mullet(*Mugil cephalus*) from different regions in Korea(<Table 1>). The fish showed the typical symptoms of vibriosis including abdominal distention, hernia, and hemorrhagic ascites. The kidney and spleen of diseased flounder were inoculated on Thiosulfate-Citrate-Bile-Sucrose (TCBS, BD, USA) agar and cultured at 25℃ for overnight. The grown-bacteria were subjected to multiplex-PCR to be identified as *V. harveyi*.(Kim et al., 2014a). Once confirmed as *V. harveyi* by the PCR, the isolated bacteria were cultivated in Brain Heart Infusion Broth(BHIB, BD, USA) containing 1.5% of NaCl for overnight. The culture was prepared as a stock by adding 20%(w/v) of glycerol and kept at -80℃ for further experiments.

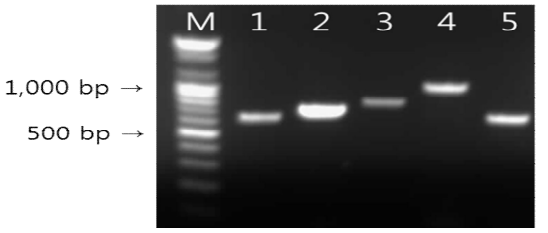
<Table 1> Isolates of *Vibrio harveyi* used in this study

Isolate code	Host	Date	Origin
E05003	red seabream(*Pagrusmajor*)	2002	Tongyeong, Korea
E05005	red seabream(*Pagrusmajor*)	2002	Tongyeong, Korea
E05006	red seabream(*Pagrusmajor*)	2002	Tongyeong, Korea
E05007	red seabream(*Pagrusmajor*)	2002	Tongyeong, Korea
E05008	black rock fish(*Sebastesinermis*)	2002	Tongyeong, Korea
E05009	rock fish(*Sebastesschlegeli*)	2002	Tongyeong, Korea
C05011	flathead mullet(*Mugilcephalus*)	2002	Tongyeong, Korea
A05022	olive flounder(*Paralichthysolivaceus*)	2003	Wando, Korea
FP4110	olive flounder(*Paralichthysolivaceus*)	2004	Jeju, Korea
FP4138	olive flounder(*Paralichthysolivaceus*)	2004	Ulsan, Korea
FP5201	rock fish(*Sebastesschlegeli*)	2005	Tongyeong, Korea
KCCM40866(=ATCC14126)	luminescing amphipod	-	Japan

2. DNA extraction and cloning

Overnight culture of the bacteria in BHIB were centrifuged at 7,000rpm for 15 min to collect bacterial cells, washed 3 times by sterilized phosphate-buffered saline(PBS) and total DNA was extracted using a genomic DNA isolation kit(High Pure PCR Template Preparation Kit, Roche Life Science, Germany) according to the instruction of manufacturer. The separated DNA was quantified by Nanovue(GE healthcare, USA) and adjusted to 0.1mg/ml.

The four OMP genes, i.e., OmpK, OmpU, OmpV and OmpW, of *V. harveyi* were amplified by PCR using specific primers for OmpW, OmpV, OmpU, OmpK of *V. parahaemolyticus*(Table 2) (Mao et al., 2007). Since those OMP genes of *V. harveyi* have not yet been reported at the time of experiment, the basic local alignment search tool(Blast)(Altschul et al., 1997) search was performed at http://blast.ncbi.nlm.nih.gov/Blast.cgi(NCBI) to find OMP gene sequences of Vibrio species and identified complete CDS of *V. parahaemolyticus* OMP genes(accession No. DQ425109).(Mao et al., 2007). Specificity of those primers in *V. harveyi* ATCC 14126 was verified by sequencing the PCR products. PCR was conducted by adding 0.5μM of each primers, 1 μl of the diluted DNA and sterile distilled water into 2x PCR premix(Bioneer, Daejeon, Korea) to make a total volume of 20 μl. PCR was performed by pre-denaturating at 94℃ for 5min and post-extended at 72℃ for 7min in 30 cycles of 94℃ for 30s, 50℃for 30s and 72℃ for 1min using a thermal cycler(PTC-220 DNA Engine Dyad Peltier, Bio-Rad, USA). The amplified products were verified by electrophoresis on a 1.5% of agarose(Sigma, USA) gel in Tris-acetate-EDTA (TAE) buffer([Fig. 1]). The PCR product was cloned into the TOPO TA Vector(Invitrogen, USA) and sequenced by Solgent co.(Korea).



[Fig. 1] PCR amplification of four OMP genes of *V. harveyi* ATCC 14126. M, 100bp DNA ladder; lane 1, OmpW; lane 2, OmpV; lane 3, OmpK; lane 4, OmpU; lane 5, OmpW with restriction sites at the 3' and 5' end.

3. Phylogenetic analysis of OMP genes

The nucleotide sequences were identified by blast searching and phylogenetic analysis of the nucleotide sequences including type strain(*V. harveyi* ATCC 14126) was performed by CLUSTALW. Phylogenetic tree was constructed using DNA sequences and the neighbour-joining method within the MEGA5 program.(Tamura et al., 2011). Node values represent percent bootstrap confidence derived from 10,000 replicates. The evolutionary distances were computed using the JTT matrix-based method and pairwise deletion option. Similarity of nucleotide sequences was analyzed by Bioedit program.

4. Structural analysis of the *V. harveyi* FP4138 OmpW protein

Since the OmpW showed no variations in the deduced amino acid sequences between different isolates, further analysis of the deduced amino acid of OmpW was carried out to identify its structure as a transmembrane channel to be used as a subunit vaccine. Signal peptide and transmembrane

domain prediction was performed by SignalP and SMART7.(Letunic et al., 2012). To build a 3D structure, the deduced amino acid sequence of *V. harveyi* FP4138 OmpW gene was subjected to protein structure homology modeling at SWISS-MODEL server(http://swissmodel.expasy.org/). Briefly, template search with Blast and HHBlits was performed against the SWISS-MODEL template library(SMTL, last update: 2014-12-12, last included PDB release: 2014-12-05). The translated amino acid sequence of *V. harveyi* FP4138 OmpW was searched with BLAST(Altschul et al., 1997) against the primary amino acid sequence contained in the SMTL. The templates of *E. coli* OmpW(SMTL ID 2f1t.1)(Hong et al., 2006) have been selected for model building. Then, a model was built based on the target-template alignment using ProMod-II.(Guex and Peitsch, 1997). Coordinates which are conserved between the target and the template are copied from the template to the model. Insertions and deletions are remodeled using a fragment library. Side chains are then rebuilt. Finally, the geometry of the resulting model is regularized by using a force field.

5. Production of recombinant OmpW protein in E. coli

The gene coding OmpW was amplified from *V. harveyi* FP4138 by PCR and produced as recombinant protein in *E. coli.*(Mao et al., 2007). We have chosen OmpW as a vaccine candidate since it showed the highest homology between the isolates. The PCR primers(<Table 2>) were designed to amplify OmpW genes coding for mature peptide and to generate the restriction sites BamHI and HindIII at the 5′ ends and 3’ end, respectively, for cloning into expression vector. The purified PCR products were digested with the restriction enzymes and ligated into pET32a vector(Novagen). The resulting recombinant plasmid pET32aOmpW were transformed into *E. coli* BL21. After verifying the constructed pET32aOmpW by restriction enzyme digestion and DNA sequencing, the recombinant *E. coli* BL21 cell was inoculated in LB medium at 37℃ with shaking-flask fermentation. When reached to logarithmic phase(OD600 = 0.6), the recombinant protein expression was induced with 1 mM isopropyl β-D-thiogalactopyranoside(IPTG).

<Table 2> Primers used for PCR amplifying OMPs of *V. harveyi*

Primer	Direction	Sequence(5'→3')[a]	Expected size(bp)	Restriction sites	Reference
OmpK-F1	Forward	ATGCGTAAATCACTTTTAGCTCTT	798	-	Mao et al., 2007
OmpK-R1	Reverse	GAACTTGTAAGTTACTGCGATGTA		-	
OmpU-F1	Forward	ATGAAAAAGACTCTAATTGCTCTT	1,008	-	
OmpU-R1	Reverse	GAAGTCGTAACGTAGACCTAGAGC		-	
OmpV-F6	Forward	CTAACGTTRACGGCTGCA	774	-	
OmpV-R6	Reverse	TACTGCCCATTGGTTTGC		-	
OmpW-F1	Forward	ATGAAAAAAACAATCTGCAGTCTA	642	-	
OmpW-R1	Reverse	GAACTTGTAACCGCCGCTGATCAT		-	
OmpW-F	Forward	CGAGGATCCATGAAAAAAACAATCTGCAG	660	*Bam*HI	
OmpW-R	Reverse	CGAAAGCTTGAACTTGTAACCGCCGCTGA		*Hind*III	

Owning to the six histidines presenting at N' end, the recombinant proteins were purified with Ni-IDA agarose resin(Novagen, Germany) according to the manual. Briefly, one liter of *E. coli* expressing each of recombinant OmpW was centrifuged for 10 min at 8,000 rpm in a centrifuge(Vision, Korea), and the pellet was resuspended in 1X binding buffer(5 mM imidazole, 0.5M NaCl, 20 mM Tris, pH 7.9). Disruption of cells was performed with an ultrasonic processor(Sonics & Materials, Inc. USA) at 30% for 10 min(a 30s sonication and 30s pause each time with cooling on ice). The supernatant was passed through 0.45μm filter(Watman, UK). The filtered cell lysates were loaded on Ni-IDA agarose resin(ABM) and washed with washing buffer(80 mM imidazole, 0.5M NaCl, 20 mM Tris, pH 7.9). Then recombinant protein was eluted with elution buffer(1M imidazole, 0.5 M NaCl, 20 mM Tris, pH 7.9). The expression and purity of the recombinant OmpW was identified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis(SDS–PAGE). The concentration of total protein in suspension was determined by using the BCA assay with bovine serum albumin as the standard protein.

6. Preparation of formalin killed cell

Formalin killed cell(FKC) was prepared by treating overnight culture of *V. harveyi* FP4138 with formalin(Merck, Germany) at the final concentration of 0.3% for 24 h at 4℃. Formalin was removed by washing 3 times with a sterile 0.01M PBS(pH 7.4) by centrifugation at 7,000 g for 15min. After final centrifugation the bacterial pellet was resuspended in PBS at the dose of 100mg/ml and stored at 4℃ until used for vaccination. To verify the inactivation of bacteria, the final solution was inoculated onto TSA containing 1.5% of NaCl, cultured at 25℃ overnight, and confirmed no colony on the agar plate.

7. Vaccination of olive flounder by the recombinant OmpW protein

The recombinant OmpW and *V. harveyi* FP4138 FKC were adjusted to 380 μg/ml and 38 mg/ml, respectively, and mixed with equal volume of Freud's incomplete adjuvant(FIA). Olive flounder with an average body weight(b.w.) of 90g were randomly divided into four groups(n=25) and intraperitoneally(i.p.) injected with 100μl of OmpW+FIA, FKC+FIA, PBS(pH 7.4) or FIA alone. Thus each fish in experimental groups was injected with 20 mg of OmpW or 20mg of FKC while fish in negative groups was injected with PBS or FIA. At three weeks post-vaccination, serum from 10 fish of each group were collected in order to measure the antibody levels employing a 96 well microplate agglutination test method and antibody titers were recorded as the dilution factor at the last well in which visible agglutination occurs. The antigen concentration used for titer assay was 5mg/ml in PBS.

8. Challenge test

At three weeks post-immunization, 15 fish from each group were challenged by i.p. injecting with live *V. harveyi* FP4138(1×10^8 cfu/fish) and then maintained in a separate tank with running seawater at 25℃ for 7 days. Cumulative mortality of each group was counted for 7 days. We have stopped challenge test at day 7 since OmpW vaccinated and control fish stopped to die after day 4 post challenge. In our previous study 7 days

were enough time to see the protection against *V. harveyi.*(Kim et al., 2014b).

9. Statistical analysis

Antibody agglutination tests were performed in triplicates and analyzed by one-way ANOVA LSD post hoc test using the SPSS software. If a significant($P < 0.05$) difference was found when comparing with negative control, it was marked with asterisk(*).

Ⅲ. Results and Discussion

1. Cloning of OMP genes

In this study, OmpK, U, W, and V genes of *V. harveyi* isolated from different fish species in different regions in Korea were partially or fully sequenced. For this, specific primers for those OMP genes on the basis of *V. parahaemolyticus* OMP gene sequences were designed and the sequences of PCR products from the type strain of *V. harveyi* ATCC 14126 were verified by comparing with chromosome sequence of a type *V. harveyi* ATCC BAA-1116 strain.

As a result, DNA sequences of cloned genes revealed that OmpK genes were 798-819bp long coding for 266-273aa long proteins with molecular weight of 27.8-30.4kDa (GenBank accession number KP659321~KP659332); OmpU genes were 1023-1041bp long coding for 341-347aa long proteins with molecular weight of 36.4-37.0kDa (GenBank accession number KP659333~KP659344); OmpW genes were homogeneously 642bp long coding for 214aa long proteins with molecular weight of 23.3kDa (GenBank accession number KP659309~KP659320). Unlike other OMPs, OmpV genes were partially cloned and were homogeneously 726bp long coding for 242aa long proteins with molecular weight of 26.2-26.3kDa (GenBank accession number KP659345~KP659356).

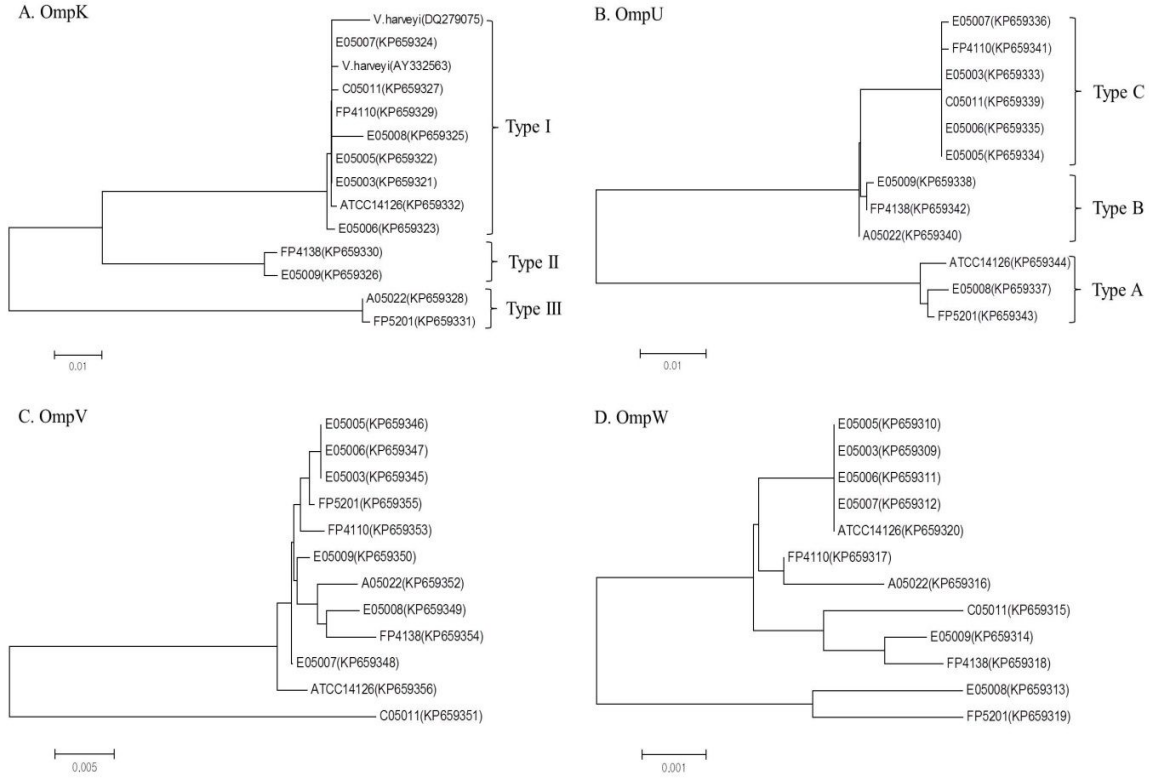
2. Phylogenetic analysis of OMP genes

Phylogenetic variations of OmpK, U, W, and V genes of *V. harveyi* isolated in this study were analyzed. Phylogenetic analysis revealed that the OMP genes from different isolates shares homology but also they are phylogenetically separated as some of them can be categorized into a few sub groups. We could have divided OmpK and U into 3 groups of type I, II, III and type A, B, C, respectively, while OmpV and W were highly homologous within the groups([Fig. 2]).

Sequence analysis of OmpK genes from 12 *V. harveyi* isolates indicated 8 isolates in group I, 2 isolates in group II and 2 isolates in group III([Fig. 2A], <Table 3>). BLAST search revealed that group I was more than 99% identical to the previously reported OmpK sequences of two other *V. harveyi* strains, i.e., EcGS020802(GenBank AY332563) and 1017(GenBank DQ279075), isolated from orange-spotted grouper and large yellow croaker, respectively, in China(Ningqiu et al., 2008).

According to OmpU sequence analysis, 12 *V. harveyi* isolates can be divided into three groups of A, B, C as 6 isolates were in group A, 3 isolates in group B and 3 isolates in group C([Fig. 2B], <Table 3>). The OmpU gene sequences of C05011, E05003, E05005, E05006 and E05007 in group A were identical.

Meanwhile, OmpV sequences were highly homologous as the eleven *V. harveyi* strains except C05011 share more than 99% similarity with each other(those of E05003, E05005 and E05006 and those of E05008 and A05022 were identical)([Fig. 2C]).



[Fig. 2] Phylogenetic tree of OmpK(A), OmpU(B), OmpV(C) and OmpW(D) genes. The tree was constructed using amino acid a multiple alignment and the neighbour-joining method within the MEGA5 program(Tamura et al., 2011). The percentage bootstrap values are shown at the branch nodes. Node values represent percent bootstrap confidence derived from 10,000 replicates. The evolutionary distances were computed using the JTT matrix-based method and pairwise deletion option. The accession number for each sequence is given after the species name and molecular type.

OmpV gene of C05011 shares only 94 or 93% of similarity with any of the OmpV from the present study or previously reported OmpV gene of *V. harveyi* strain NBRC15634(=ATCC 14126, GenBank KF111419) in blast search, respectively (<Table 3>).

The OmpW sequences of the 12 *V. harveyi* isolates were highly homologous and share more than 99% similarity with each other(those of ATCC 14126, E05003, E05005, E05006 and E05007 were identical)([Fig. 2D], <Table 3>). Although 1 or 2 nucleotides were different in some strains, the deduced amino acid sequences were completely homologous. This is indicating that OmpW can be also used for the diagnosis of *V. harveyi* infection. In the previous studies, besides their potentials as vaccine candidates, OMP genes have been proved to be good targets for rapid identification of Gram negative bacteria. Detection methods targeting OMP genes have been developed in fish pathogenic bacteria including *V. parahaemolyticus* and *V. alginolyticus* (Marhual et al.,2012), and *Aeromonas*

<Table 3> Nucleotide sequence similarity of OmpK and OmpU genes from *V. harveyi* isolates

Gene	Isolate	14126*	A05022	C05011	E05003	E05005	E05006	E05007	E05008	E05009	FP4110	FP4138	FP5201
OmpK	**14126***	100											
	A05022	81.4	100										
	C05011	97.8	82.9	100									
	E05003	97.9	83.1	99.9	100								
	E05005	97.9	83.0	99.9	100.0	100							
	E05006	97.7	82.7	99.6	99.7	99.7	100						
	E05007	97.9	83.1	99.9	100.0	100.0	99.7	100					
	E05008	97.3	82.8	99.2	99.4	99.4	99.1	99.4	100				
	E05009	87.7	81.8	86.2	86.4	86.4	86.2	86.4	85.8	100			
	FP4110	97.9	83.1	99.9	100.0	100.0	99.7	100.0	99.4	86.4	100		
	FP4138	89.4	85.1	89.6	89.7	89.7	89.6	89.7	89.1	99.4	89.7	100	
	FP5201	81.2	99.9	82.8	82.9	82.9	82.6	82.9	82.7	81.6	82.9	84.7	100
OmpU	**14126***	100											
	A05022	87.3	100										
	C05011	89.1	97.5	100									
	E05003	89.1	97.5	100.0	100								
	E05005	89.1	97.5	100.0	100.0	100							
	E05006	89.1	97.5	100.0	99.9	99.9	100						
	E05007	89.0	97.4	99.9	99.9	99.9	99.9	100					
	E05008	97.0	89.3	86.9	86.9	86.9	86.9	86.8	100				
	E05009	87.1	99.8	97.3	97.3	97.3	97.3	97.2	89.0	100			
	FP4110	89.0	97.4	99.9	99.9	99.9	99.8	99.8	86.8	97.2	100		
	FP4138	87.2	99.9	97.4	97.4	97.4	97.4	97.3	89.2	99.9	97.3	100	
	FP5201	97.0	89.5	87.1	87.1	87.1	87.1	87.0	99.6	89.3	87.0	89.4	100

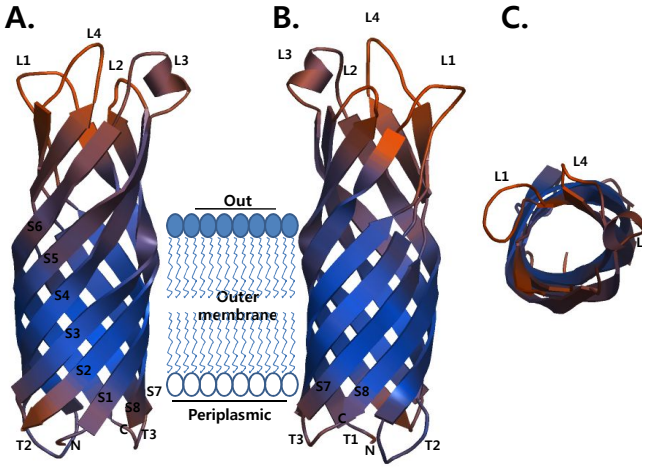
* ATCC 14126

hydrophila (Maiti et al., 2009). A one-step multiplex PCR assay detecting OmpW and a regulatory protein(ToxR) for rapid species-specific identification was developed to detect human pathogenic *V. cholera* and found that primers for OmpW were more specific than primers for ToxR(Nandi et al., 2000). Thus the present study can provide basic data on genetic variations of major OMP genes in Omp for the development of future diagnosis methods for *V. harveyi* by detecting the OMP genes.

Full length of OmpW gene was sequenced from *V. harveyi* FP4138 to be used in vaccination study. The OmpW sequences of the 12 Korean *V. harveyi* isolates shares 92.5% of identity with previously reported OmpW(GenBank EEZ89828) of *V. harveyi* 1DA3.

3. Analysis of the *V. harveyi* FP4138 OmpW protein

The ORF sequence of OmpW is comprised of 642 nucleotides encoding a protein of 214 amino acids with a calculated molecular mass of 23 kDa. SignalP and SMART 7 predicted that the protein consists of a signal peptide (MKKTICSLAVVAA LVSPSVFA, 1-21aa) and transmembrane domains (22-214aa). To estimate the structure of OmpW protein, protein homology modeling was performed at http://swissmodel.expasy.org/. As a result, the SWISS-MODEL template library(SMTL version 2014-12-12, PDB release 2014-12-05) was searched with Blast(Altschul et al., 1997) and HHBlits(Remmert et al., 2012) for evolutionary related structures matching the target sequence of *V. harveyi* FP4138 OmpW and found 94 templates including OmpW, OmpX, OmpF, OmpOPRG, long-chain fatty acid transport proteins, etc. Among the OmpW templates, *E. coli* OmpW(SMTL ID 2f1t.1)(Hong et al., 2006) was chosen for modeling with the highest Qmean score. The resulting structure demonstrated that *V. harveyi* FP4138 OmpW is composed with 8 β-strands(S1-S8), 4 extracellular loops(L1-4) and 3 periplasmic turns(T1-3)(Fig. 3A and B). View of OmpW from top indicated the ellipsoid shape of the barrel([Fig. 3C]).

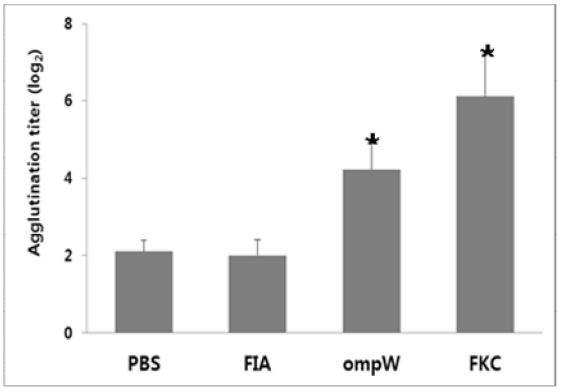


Target MKKTICSLAVVAALVSPSVFAHSEGDFILRVGAASVVPNDSSDKILGSQEELKVDSNTQLGLTFGYMFTDNISLEILAAT
2f1t.1.A ----------HEAGEFFMRAGSATVRPTEGAGGTLGSLGGFSVTNNTQLGLTFTYMATDNIGVELLAAT
Target PFSHDISDLLGLGDIADTKHLPPTVMLQYYFGDSQSKFRPYVGAGLNYTMFFDEGFNGKAKDVGLTDLKLDDSFGLAANV
2f1t.1.A PFRHKIGTR-ATGDIATVHHLPPTLMAQWYFGDASSKFRPYVGAGINYTTFFDNGFNDHGKEAGLSDLSLKDSWGAAGQV
Target GVDYMINESWFLNASAWYANIETEATYKFNGAAQKTDVKINPWVFMISGGYKF
2f1t.1.A GVDYLINRDWLVNMSVWYMDIDTTANYKLGGAQQHDSVRLDPWVFMFSAGYRF

[Fig. 3] Schematic structure of the predicted *Vibrio harveyi* FP4138 outer membrane protein (OmpW) protein generated by SWISS-Model based on a template structure of E. coli OmpW(SMTL ID 2f1t.1).(Hong et al., 2006). Ribbon diagrams(A and B) viewed from the side, with B, 180° rotated relative to A. Extracellular loops(L1-4) and periplasmic turns(T1-3) are indicated. The N terminus in B is indicated with N and the C terminus is indicated with C. The β-strands are numbered from S1 to S8. The approximate boundary of the hydrophobic part of the outer membrane is indicated with horizontal lines. View of OmpW from top(C) shows the ellipsoid shape of the barrel.

4. Vaccination of olive flounder by the recombinant OmpW protein produced in *E. coli*

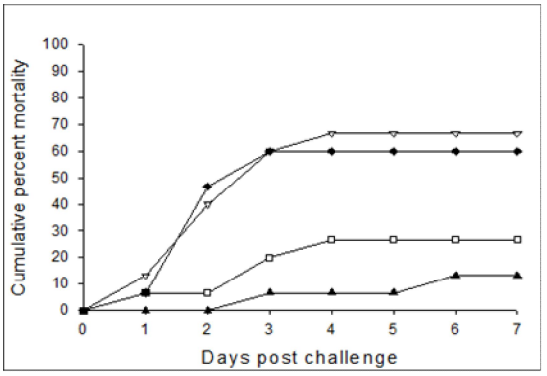
After vaccination with the recombinant OmpW and FKC of *V. harveyi,* the agglutinating titers of serums produced in olive flounder were measured. At three weeks post-vaccination, log2 titers in the sera of immunized flounders reached at above 5.5, and the maximum reached at 10, while log2 titers in negative control serum had only 2. Antibody titer was 4.2+0.7(mean+SEM, n=9) in OmpW vaccinated fish and 6.1+1.1(n=9) in FKC vaccinated fish(positive control) while 2.1+0.3(n=10) and 2.0+0.4(n=9) in PBS and FIA injected fish(negative controls), respectively([Fig. 4]). The results indicated that the specific antibody titer in the sera of OmpW or FKC immunized flounder were significantly higher than in control serum ($p<0.05$).



[Fig. 4] Antibody agglutination titers after vaccination with recombinant OMPW (200 mg/kg) and FKC (20 mg/kg) of *V. harveyi* FP4138 in combination with equal volume of FIA. Negative control fish was injected with PBS or FIA. At three weeks post-vaccination, sera from 10 fish of each group were collected and antibody agglutination tests were performed in triplicates. Antibody titers were recorded as the dilution factor at the last well in which visible agglutination occurs and significant ($P < 0.05$) difference was marked with asterisk (*) when comparing with negative controlby one-way ANOVA LSD post hoc test using the SPSS program (n=10).

5. Challenge test

In challenge test, fish started to die from day 1 in OmpW vaccinated and control groups, stopped to die from day 4 and sustained until day 7 while FKC vaccinated fish started to die from day 3 and the mortality was gradually increase until day 7. Dead fish represented serious typical symptoms of vibriosis, i.e., an acute bacterial haemorrhagic septicaemia like ulceration and haemorrhage of skin and fins, exophthalmia, abdominal distension, and hyperaemia of gills and intestines. The mortalities of vaccinated groups(13.3% for FKC group and 26.7% for OmpW group) were significantly lower than the control groups(60% for PBS group and 66.7% for FIA group) ([Fig. 5]). Relative percent surviva (RPS=(1−[% Mortality of vaccinated group / % Mortality of control group 4]) × 100) of OmpW and FKC vaccinated fish reached at 60% and 80%, respectively, indicating that olive flounder was well protected against infection with *V. harveyi* after vaccination with OmpW. This RPS of OmpW vaccinated group is slightly lower than 71% of RPS in the study by Yu et al.(2013). They have done the challenge test at one month after vaccination with 15 μg/100 μl of OmpW. The size of fish was about 15g much smaller than in our study which is about 90g. Although OmpW of *V. harveyi* T4D and OmpW in this study share same homology of 92 % to OmpW (GenBank EEZ89828) of *V. harveyi* 1DA3, it was not



[Fig. 5] Cumulative mortality curves for olive flounder at 7 days after challenge with *V. harveyi* FP4138 (1×10^8cfu/fish)at three weeks post-immunization with recombinant OMPW(-□-), FKC(-▲-),control(PBS)(-▽-) and control (FIA) (-◆-). Relative percentage survival (RPS) = (1−[% Mortality of vaccinated group / % Mortality of unvaccinated negative control group (PBS)])×100 (n=15).

possible to calculate the sequence similarity between OmpW of *V. harveyi* T4D and OmpW in this study since we could not find the sequence of OmpW of *V. harveyi* T4D from NCBI and EMBL databases.

OmpW of other fish pathogenic bacteria including *V. parahaemolyticus*(Mao et al., 2007) and *V. alginolyticus*(Qian et al., 2007) was reported to be highly immunogenic and able to induce protective immunity in fish like large yellow croaker. Indeed OmpW is known to be involved in protection of bacteria against various environmental stress like osmosis(Xu et al., 2005), oxidation(Gil et al., 2007), temperature and the lack of nutrients and oxygen.(Nandi et al., 2005). Proteomic analysis revealed that OmpW is related to the resistance against antibiotics such as ampicillin, tetracycline and ceftriaxone. The upregulation of OmpW is also correlated with an increase in bacterial virulence (Goel et al., 2010). Thus the raised antibody against OmpW may have inhibited the pathogenicity of *V. harveyi* and protect fish from the death caused by *V. harveyi* infection.

To summarize, OmpK and U genes of *V. harveyi* isolated from marine fishes in Korea had genetic variations while OmpW gene was highly conserved in the samples analyzed in this study. Protein homology modeling using the deduce amino acid sequence of *V. harveyi* FP4138 OmpW demonstrated the structure of the OmpW with 8 β -strands, 4 extracellular loops and 3 periplasmic turns, revealing the ellipsoid shape of the barrel. Vaccination and challenge study showed that the recombinant OmpW might be related to the protection against Korean isolates of *V. harveyi* in olive flounder.

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