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Expression, Characterization and Immunogenicity of a Major Outer Membrane Protein from *Vibrio alginolyticus*

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Abstract *Vibrio alginolyticus* is one of the *Vibrio* pathogens common to humans and marine animals. During infection and induction of the host immune response, outer membrane proteins of bacteria play an important role. In this study, an outer membrane protein gene (*ompW*) was cloned from *V. alginolyticus* and expressed in *Escherichia coli*. The 645 bp open reading frame (ORF) encodes a protein of 214 amino acid residues with a predicted molecular weight of 23.3 kDa. The amino acid sequence showed a high identity with that of *Photobacterium damselae* (96.2%) and *Vibrio parahaemolyticus* (94.4%). The alignment analysis indicated that OmpW was highly conserved. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that the gene was over-expressed in *E. coli* BL21(DE3). Western blot analysis revealed that the expressed protein had immunoreactivity. The recombinant protein was purified by affinity chromatography on Ni-NTA Superflow resin. Large yellow croaker vaccinated with the purified OmpW showed significantly increased antibody to OmpW, which could resist the infection by *V. alginolyticus*. A specific antibody was detected by enzyme-linked immunosorbent assay. This study suggested that the conserved OmpW could be an effective vaccine candidate against infection by *V. alginolyticus*.

Key words *Vibrio alginolyticus*; *ompW*; expression; characterization; immunogenicity; vaccine candidate

In gram-negative pathogenic bacteria, the outer membrane plays an important role in infection and pathogenicity to the host [1]. It is basically composed of protein, lipid and sugar, which could be easily recognized as foreign substances by the host's immunological defense systems. Of these components, membrane proteins play a crucial role in many cellular and physiological processes, and are particularly attractive for the development of vaccines and diagnostic kits [2,3]. Recent studies emphasize the role of outer membrane proteins (OMPs) of pathogenic bacteria in protective antigenicity [4,5]. OmpW belongs to a family of small OMPs that are widespread in gram-negative bacteria. It forms an eight-stranded β-barrel with a long and narrow hydrophobic channel [6,7], which is involved in osmoregulation [8,9] and transportation of small hydrophobic molecules across the bacterial outer membrane [10]. In *Photobacterium damselae*, OmpW is required for environmental salt (NaCl) regulation [11]. The expression of *ompW* is down-regulated upon σ^E regulon activation [12], and the modulation of expression depends on environmental factors such as temperature, salinity and oxygen [13]. OmpW is a major antigen in bacterial infections and has been shown to be immunogenic in *Vibrio cholerae* [14,15].

Vibrio alginolyticus, a halophilic gram-negative bacterium that inhabits marine and estuarine environments, is one of the major human *Vibrio* pathogens [16]. It causes syndromes of clinical illness including wound infections, otitis media, food intoxication, gastroenteritis and septicemia [17,18]. Furthermore, it is one of the main *Vibrio*

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pathogens affecting marine animals, such as marine fish, shrimp and shellfish [19,20], which leads to large economic damage. Thus, it is essential to explore an effective protective pathway against infection by this microorganism.

In this study, *ompW* was cloned from *V. alginolyticus* strain ZJ04107 and expressed in *Escherichia coli*. The purified protein was evaluated to assess if it could serve as an effective vaccine candidate against *V. alginolyticus*.

Materials and Methods

Bacterial strains

V. alginolyticus strain ZJ04107 was isolated from pathogenic large yellow croaker (*Pseudosciaena crocea*) cultured in marine cages in Xiangshan Bay, Zhejiang Province, China. Another *V. alginolyticus* strain, which served as the typical strain, was purchased from the Microbiology Institute of the Chinese Academy of Sciences (Beijing, China). Both strains were cultured on thiosulfate-citrate-bile salt-sucrose (TCBS) agar plates (Tianhe, Hangzhou, China), then maintained in ZoBell2216E broth at −70 ºC.

Cloning of *ompW*

The primers (P1, 5′-ATGAAAAAAACAATCTGCAGTC-3′ and P2, 5′-TTAGAACTTGTAACCGCCGCTG-3′) were designed according to the most conserved region of *ompW* from other *Vibrio* species in GenBank (**Fig. 1**). Polymerase chain reaction (PCR) was carried out with the template genomic DNA from *V. alginolyticus* strain ZJ04107. The PCR product was purified with a PCR purification kit (Sangon, Shanghai, China) and subcloned into pGEM-T-easy vector (Promega, Wisconsin, USA) for sequencing.

Construction of recombinant expression plasmid

The primers (P3, 5′-GCGAATTCGCAGTGGTTGCT-GCACTCGT-3′, and P4, 5′-CG CTCGAGTTAGAACTT-GTAACCGCCGCT-3′) with specific restriction sites (*Bam*HI/*Xho*I) were designed from the cloned nucleotide

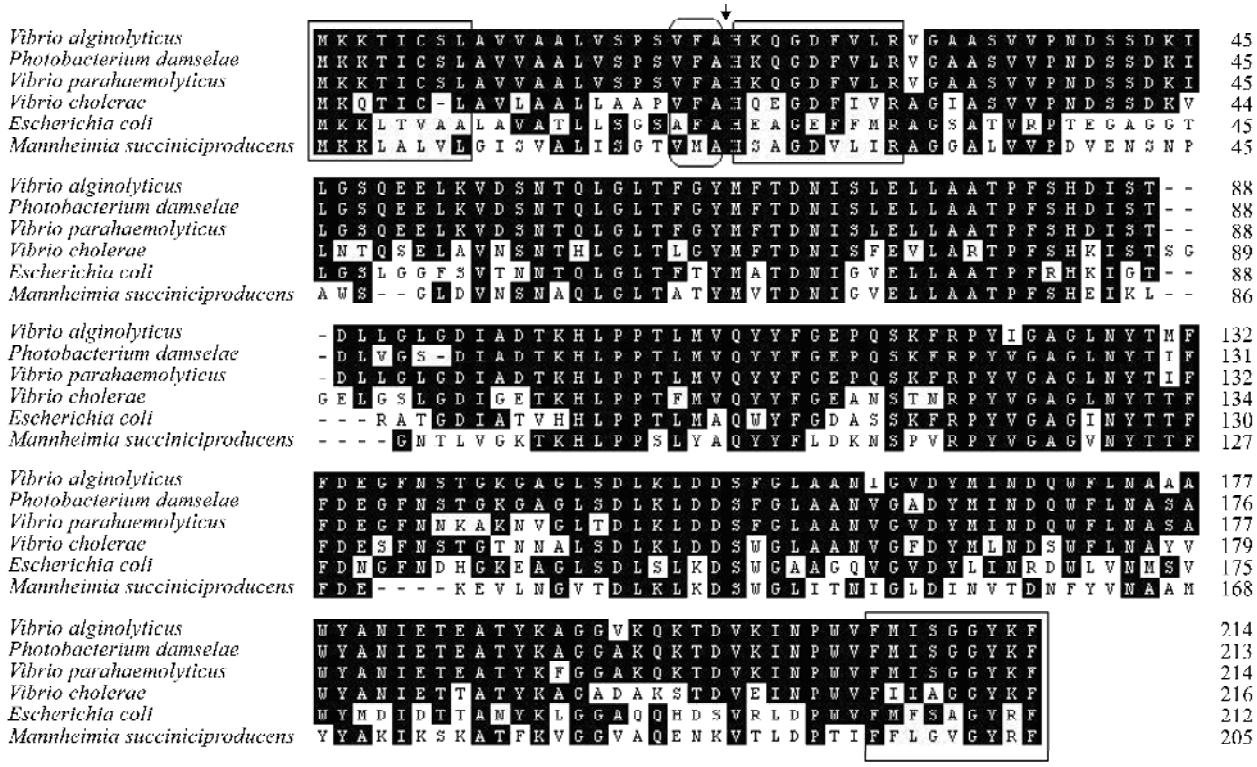

Fig. 1 Alignment of OmpW sequence from *Vibrio alginolyticus* and other strains (listed in Table 1)

The amino acid sequences were aligned using the ClustalW program from the European Bioinformatics Institute (Cambridge, UK). Identical amino acid residues presented are boxed in black, and boxes in gray indicate the location of similar residues. Alignment gaps are indicated by (–). The arrow indicates a signal-sequence peptide cleavage site. V-X-A is indicated with an elliptic bracket. The regions used to design primers are indicated with vertical brackets.

sequence to amplify the encoding sequence for the mature OmpW protein, and incorporated into the PCR. The purified PCR products were digested with *Bam*HI and *Xho*I, and subcloned into pET-30a(+) vector (Novagen, Darmstadt, Germany), which was digested with the same endonucleases. The resultant plasmid named pET-*ompW* was detected by restriction enzyme analysis and sequencing.

Expression and purification of recombinant protein

The overnight cultures of BL21 (DE3) (Novagen) harboring recombinant plasmid pET-*ompW* were diluted 1:100 (*V*/*V*) in fresh Luria Broth with kanamycin (50 μg/ml), then incubated at 37 ºC until the optical density at 600 nm (A_{600}) reached 0.6. The protein expression was induced with 1 mM isopropyl-β-*D*-thiogalactopyranoside (IPTG) (BBI, Colorado, USA) for 5 h at 37 ºC after the optimization of expression conditions.

Bacterial cells were harvested by centrifugation, then washed and resuspended in buffer A (10 mM Tris-HCl, 200 mM NaCl, pH 8.0). The cell suspension was disrupted by sonication in an ice bath (350 W, 3×10 min) and centrifuged (12,000 *g* for 20 min at 4 ºC). The pellet containing inclusion bodies of recombinant protein was washed, then dissolved in buffer A containing 8 M urea. The recombinant protein was subsequently purified by affinity chromatography on Ni-NTA Superflow resin (Pierce, Boston, USA) according to the manufacturer's instructions. The bound protein was eluted with buffer A containing 8 M urea and 300 mM imidazole. The purified recombinant protein in the denatured form was renatured using stepwise dialysis against decreasing concentrations of urea (6 M to nil) taken in buffer A containing 1% (*V*/*V*) Triton X-100. Finally, the protein solution was further dialysed against buffer A containing decreasing concentrations of the detergent. The protein concentration was determined by the Lowry method. The purified protein was stored at −40 ºC before use.

Anti-sera

Rabbit anti-sera were used to detect the recombinant protein. Two male adult New Zealand White rabbits were intramuscularly immunized with the purified OmpW recombinant protein (300 μg each rabbit) emulsified with Freund's complete adjuvant. After 4 weeks of the initial immunization, another dose of the purified protein in Freund's incomplete adjuvant (100 μg per dose) was given. Two weeks later, blood was collected from the rabbits, then clotted at room temperature for 1 h and stored at 4 ºC overnight. After centrifugation, the separated rabbit anti-OmpW anti-sera were stored at −70 ºC.

By gel filtration and ion exchange chromatography, an antibody (IgM) fraction was obtained from the serum of large yellow croaker anti-ZJ04107 [21]. The IgM fraction was mixed with Freund's incomplete adjuvant and injected subcutaneously into a rabbit. The rabbit anti-fish serum was separated as described above.

Western blotting analysis

The expression of recombinant OmpW protein was examined by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The immunoactivity was analyzed by Western blotting according to the standard protocol [22]. After SDS-PAGE, protein bands were electroblotted onto a nitrocellulose membrane (Hybond-C; Amersham, New Jersey, USA) that was treated with blocking solution (5% non-fat milk) for 2 h. After washing three times (15 min each time) with TBST (TBS with 0.05% Tween-20), the membrane was incubated with the rabbit anti-OmpW serum (1:800) with gentle agitation for 1 h, then further incubated for 1 h with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibodies (Dingguo, Beijing, China) diluted at 1:1000. After thorough washing with TBST, the membrane was dipped in the HRP tetramethylbenzidine liquid membrane substrate (Amresco, Solon, USA) for color development.

Fish

Large yellow croakers (*P. crocea*) with an average weight of 50 g were bought from a seawater cage aquaculture farm in Ningbo, China. The fish were held in 50 m^3 aquaria supplied with running sand-filtered and ultraviolet-treated seawater at 28 ºC. The salinity, pH and dissolved oxygen were maintained at 35 ppt, 8.0 and 7.5 mg/L, respectively. The fish were fed twice daily with 3–5 mm dry pelleted diet (10% carbohydrate, 20% lipid, 55% protein and 12% ash) during the acclimatization and experimental periods.

Vaccination

Large yellow croakers were randomly divided into groups of 75 fish and acclimatized for one week. The fish were anesthetized by immersion in a 50 mg/L solution of tricaine methane sulfonate (MS-222). During the experiment, three replica groups of 75 fish per treatment were assessed where the treatment groups were intraperitoneally injected with 200 μg OmpW per fish in 200 μl sterile PBS and the negative control group was intraperitoneally injected with 200 μl PBS alone. At week 3, 4, 5, 6 and 7 after vaccination, five fish in each treatment group

were bled from the caudal vein, and the sera were prepared for specific antibody titer detection.

Challenge and evaluation of immunoprotection

Seven weeks post-vaccination, the fish in each group (50 per group) were used for the challenge test. Bacterial suspension of *V. alginolyticus* strain ZJ04107 was prepared and adjusted to a cell density of approximately 1×10^9 cells/ml. In a preliminary assay, the median lethal dose (LD_{50}) was determined to be approximately 2.0×10^7 cfu per fish. The fish were anesthetized and challenged by intraperitoneal inoculation with 100 μl of 1×10^9 cells/ml (1.0×10^8 cfu per fish) cell suspension. Vaccine efficacy was determined by the relative percent survival (RPS) and histopathological damage among experimental treatments. Cumulative mortality and clinical signs were recorded daily for three weeks post-challenge, and dead fish were autopsied to determine the cause of death. The presence of *V. alginolyticus* in the tissues was determined by bacterial culture in TCBS agar. The RPS was calculated as previously described [23]:

RPS=1–(%mortality of vaccinated group/%mortality of control group)×100

The significant difference was calculated by Fisher's exact test.

Enzyme-linked immunosorbent assay (ELISA)

Five serum samples from each of the treatments and the control were assayed for antibody response against OmpW by ELISA. The 96-well ELISA plates were coated overnight at 4 ºC with the purified OmpW antigen (4 μg/ml diluted in the preliminary assay) in 100 μl carbonate coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6). The plates were washed three times with PBS containing 0.05% Tween-20, then blocked with 5% skimmed milk in PBS containing 0.02% NaN_3 for 1 h at 37 ºC. After washing three times with PBST, 2-fold serial dilutions of the fish anti-OmpW sera were added to the triplicate wells of the plates and incubated for 90 min at 37 ºC. The plates were washed three times, then added with rabbit anti-fish serum (1:2000) and incubated for 2 h at 37 ºC. After washing three times, the plates were added with HRP-conjugated goat anti-rabbit IgG (1:1000) (Dingguo) and incubated for 2 h at 37 ºC. After washing, a coloring reagent *o*-phenylenediamine substrate buffer was added and incubated for 20 min at 37 ºC, then 2 M H_2SO_4 was added to stop the reaction. The plates were then read at 492 nm with a microplate reader (Bio-Rad, California, USA). The significant difference was calculated by Student's *t* test.

Results and Discussion

Cloning and characterization of *ompW*

By PCR and sequencing, a 645 bp full-length ORF of *ompW* was obtained from the prepared genome DNA of *V. alginolyticus* strain ZJ04107 (**Fig. 2**). The deduced amino acid sequence of *ompW* consists of 214 amino acid residues with a predicted molecular weight of 23.3 kDa (GenBank accession No.: DQ075316).

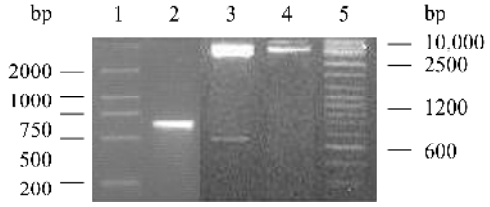

Fig. 2 Electrophoresis identification of cloned *ompW* by polymerase chain reaction and detection of pET-*ompW* recombinant plasmid by restriction enzyme analysis

1, DNA molecular marker (DL2000); 2, 645 bp amplified fragment of *ompW*; 3, the detection of pET-*ompW* recombinant plasmid digested with *Bam*HI and *Xho*I; 4, pET-30a(+); 5, GeneRuler DNA Ladder Mix.

Using Blastn in GenBank (http://www.ncbi.nlm.nih.gov/BLAST), *ompW* of *V. alginolyticus* was found to have high similarity with other *Vibrio* species (**Table 1**). The amino acids identities were 96.2% with *Ph. damselae*, 94.4% with *V. parahaemolyticus*, 70.6% with *V. cholerae* and 57.1% with *E. coli*, which indicated that OmpW was highly conserved. The highly conserved domain of OmpW was also indicated in the alignment analysis (**Fig. 1**).

Table 1 Strains and GenBank accession numbers of the *OmpW* used for alignment analysis

Strains for *OmpW* *	GenBank accession No.
V. alginolyticus	DQ075316
V. parahaemolyticus	DQ425109
V. cholerae	X51948
E. coli	CP000247
P. damselae	DQ251175
M. succiniciproducens	AE016827

* *V.*, *Vibrio*; *E.*, *Escherichia*; *P.*, *Photobacterium*; *M.*, *Mannheimia*.

Analyzed by SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP-3.0/), the ORF of OmpW includes a 21 amino acid signal peptide in the N-terminal domain, which has characteristics common to bacterial signal peptides [24]. Signal peptides possess a consensus cleavage site for a signal peptidase, at positions −3 to −1, A (G/S/L/T/V/C)-X-A is the most commonly observed sequence [25, 26]. In **Fig. 1**, the arrow indicates a signal-sequence peptide cleavage site, the amino acids at positions −3 to −1 are V(A)-F(M)-A, which is consistent with the observed sequence V-X-A of the signal peptides of transmembrane OMP. Characterized with a β-barrel structure, the transmembrane OMPs can translocate across the cytoplasmic membrane by the secretion machinery, cleave their signal peptides by leader peptidase (Lep) and arrive at the outer membrane [27,28].

Expression and measurement of the recombinant OmpW

After detection by restriction enzyme analysis and sequencing, the constructed recombinant expression plasmid pET-*ompW* was introduced into *E. coli* BL21(DE3). The negative control was the cells transformed with pET-30a(+) vector alone. The preliminary experiments showed that the induced recombinant cells started expression at 2.5 h and reached a maximum level at 4–6 h. Thus, the optimal time for the expression was 5 h after IPTG induction. As it was shown in **Fig. 3**, the recombinant protein was analyzed by SDS-PAGE with 12% SDS polyacrylamide gel. The results showed that OmpW recombinant protein gained over-expression in *E. coli* BL21(DE3) [**Fig. 3(A)**]. The antigenicity of the recombinant fusion protein was also shown by the immunized rabbit anti-OmpW serum. The rabbit antiserum recognized the recombinant protein OmpW and the OmpW present in the whole cell lysates of *V. alginolyticus* [**Fig. 3(B)**].

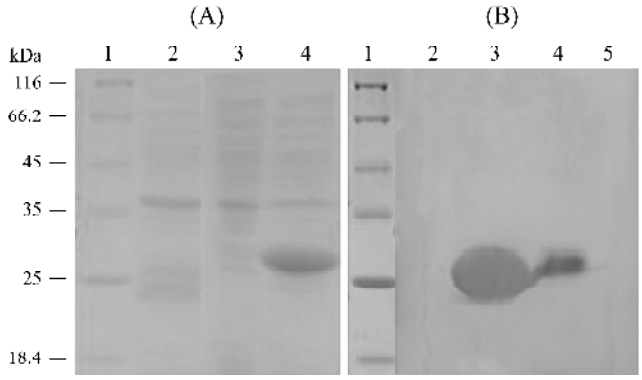

Fig. 3 SDS-PAGE and Western blotting analysis of pET-OmpW expression

(A) SDS-PAGE of pET-*ompW* expression in *Escherichia coli* BL21 (DE3). Lane 1, protein molecular marker; Lane 2, pET-30a(+) with isopropyl-β-*D*-thiogalactopyranoside (IPTG) induction; Lane 3, pET-*ompW* without IPTG induction; Lane 4, pET-*ompW* with IPTG induction. (B) Western blotting analysis of pET-*ompW* expression. Lane 1, protein molecular marker; Lane 2, pET-*ompW* without IPTG induction; Lane 3, pET-*ompW* with IPTG induction; Lane 4, the whole cell lysates of *Vibrio alginolyticus* ZJ04107; Lane 5, pET-30a(+) with IPTG induction.

Vaccination and challenge

The immune response of the fish was assessed by ELISA at weeks 3, 4, 5, 6 and 7 after vaccination. The results indicated that a specific antibody titer against OmpW was detected in all sera of the vaccinated large yellow croakers (P<0.05) (**Fig. 4**). The fish immunized intraperitoneally with OmpW produced a high level of anti-OmpW antibody. The antibody level was statistically significant in the vaccinated fish in relation to the negative control. Antibodies could be detected at week 3 after vaccination, and the antibody titer reached its highest level at week 5. The control test showed a decrease of absorbance according to the dilution test of the antigen and antibody.

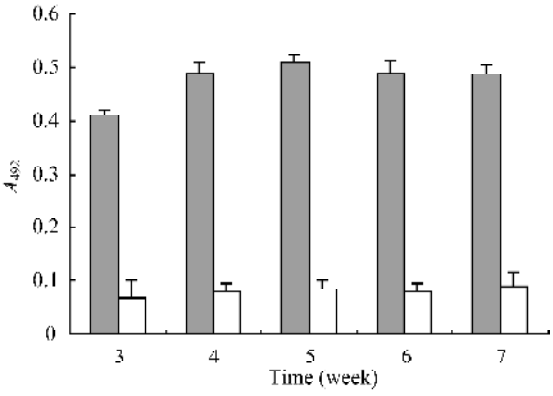

Fig. 4 Specific antibody titers by enzyme-linked immunosorbent assay in large yellow croaker sera at weeks 3, 4, 5, 6 and 7 after vaccination with OmpW

The five high columns are the average absorbed at 492 nm of vaccinated groups respectively at weeks 3, 4, 5, 6 and 7; the low columns are those of unvaccinated groups. Significant differences from the control groups were P<0.05.

Vaccinated with OmpW, the fish obtained a certain level of protection. The results showed that the fish survival rate was significantly higher in the treatment groups than the control group after challenge with *V. alginolyticus*

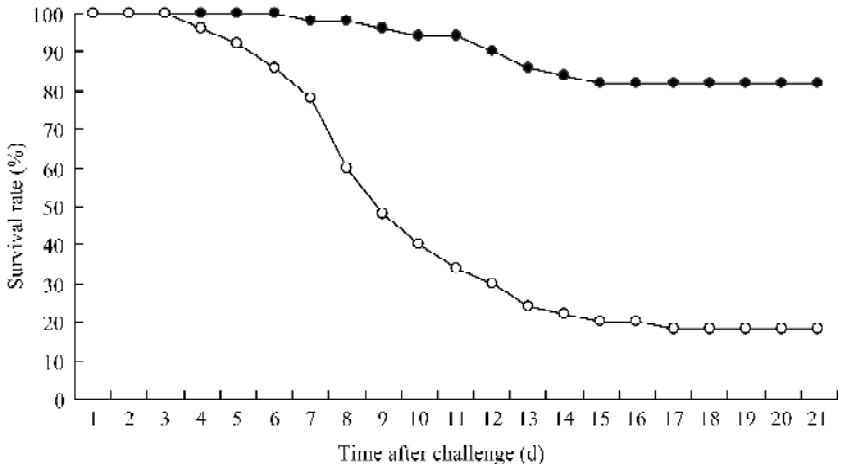

Fig. 5 Survival rate of large yellow croakers after challenge with *Vibrio alginolyticus* ZJ04107

Significant difference from the control group was obtained ($P< 0.05$). Relative percent survival calculated as RPS=1−(%mortality of vaccinated group/% mortality of control group)×100.

ZJ04107 (**Fig. 5**). On day 4, the fish in the control group began to die, with a sudden increase in the death rate at day 6−11, then a decrease until day 17. However, the survival rate in the vaccinated group was significantly higher than the unvaccinated group. The RPS reached 78% in the former group, which indicated that after vaccination with OmpW, large yellow croakers were well protected against infection with *V. alginolyticus*. The histopathological analysis of the vaccinated and non-vaccinated fish that survived the bacterial challenge revealed signs of tissue lesions resulting from *V. alginolyticus* infection. Major lesions were observed in the liver, spleen and kidney including severe necrosis and hemorrhagic infiltration (data not shown).

V. alginolyticus is one of the main *Vibrio* pathogens in marine animals, and leads to large economic damage in marine aquaculture. It is necessary to develop an effective vaccine against this organism. Vaccination with OMPs has been shown to be effective against infection by *Aeromonas salmonicida*, *V. anguillarum* and *A. hydrophila* in fish [4,5]. This study showed that the conserved OmpW is an effective vaccine candidate against infection by *V. alginolyticus*. Research into whether the conserved OmpW could also serve as a useful vaccine candidate against other pathogenic *Vibrios* is ongoing.

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