Protection against *Vibrio alginolyticus* in crimson snapper *Lutjanus erythropterus* immunized with a DNA vaccine containing the *ompW* gene

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ABSTRACT: The outer membrane proteins of *Vibrio alginolyticus* play an important role in the virulence of the bacterium and are potential candidates for vaccine development. In the present study, the *ompW* gene was cloned, expressed and purified. A DNA vaccine was constructed by inserting the *ompW* gene into a pcDNA plasmid. Crimson snapper *Lutjanus erythropterus* (Bloch) were injected intramuscularly with the recombinant plasmid pcDNA-*ompW*. The expression of the DNA vaccine was detected in gill, head kidney, heart, liver, spleen and injection site muscle of crimson snapper by RT-PCR 7 and 28 d post-vaccination. The ELISA results demonstrated that the DNA vaccine produced an observable antibody response in all sera of the vaccinated fish. In addition, crimson snapper immunized with the DNA vaccine showed a relative percentage survival (RPS) of 92.53 %, indicating effective protection against *V. alginolyticus* infection.

KEY WORDS: Vibriosis · Vaccination · Recombinant plasmid · Outer membrane protein · pcDNA · Aquaculture

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INTRODUCTION

Vibriosis is an important disease causing high mortality in marine aquatic animals (Austin 1984). *Vibrio alginolyticus* is a Gram-negative bacterium of the family *Vibrionaceae* and is one of the major *Vibrio* pathogens endangering the aquaculture of marine animals in the coastal provinces of south China (Cai et al. 2007, Liang et al. 2011). The commercially important crimson snapper *Lutjanus erythropterus* (Bloch) is widely distributed throughout the Indian Ocean and the tropical parts of the western Pacific Ocean, ranging from India through the entire Malay Archipelago to China, the Philippines, and Australia (Zhang et al. 2006). *L. erythropterus* is considered to

be a good species for aquaculture because of its robustness in heavily populated conditions and its rapid growth at elevated temperatures. Cultured *L. erythropterus* suffered serious production losses due to *V. alginolyticus* in China (Cai et al. 2010).

Control of vibriosis through antimicrobial treatment is not always successful and could be hazardous for the environment since resistant bacteria may be selected and the resistance transferred to aquatic animal or human pathogens. Therefore, it is essential to develop alternative effective prophylaxis against *Vibrio* infections. Compared with antimicrobial treatment, vaccination is a safer strategy for preventing vibriosis (Khushiramani et al. 2007, Cai et al. 2010). However, due to the diversity of the pathogens

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and their serotypes, progress in the development of vaccines against vibriosis has been slow. Therefore, more effort is required to develop a safe and effective vaccine (Sommerset et al. 2005, Li et al. 2010b).

Recently, DNA vaccines composed of plasmids equipped with a eukaryotic expression cassette have proven promising against viral or bacterial infections in fish, including infectious hematopoietic necrosis virus, viral haemorrhagic septicaemia virus, *Aeromonas veroni*, *Vibrio anguillarum*, *V. harveyi* and *V. parahaemolyticus* (Boudinot et al. 1998, Corbeil et al. 1999, Vazquez-Juarez et al. 2005, Kumar et al. 2007, Hu & Sun 2011, Liu et al. 2011).

Outer membrane proteins (OMPs) are important for adhesion to host cells and are highly immunogenic bacterial components. They are easily recognized as foreign substances by the host's immunological defence system (Qian et al. 2007, Li et al. 2010a) and some studies have emphasized the role of OMPs of pathogenic bacteria in protective antigenicity because of their exposed epitopes on the cell surface (Karalus & Murphy 1999). With regard to *Vibrio* species, attention has been paid to several OMPs such as OmpW, OmpK, OmpU and OmpV. The OmpW is a member of the small molecular OMP family of Gram-negative bacteria, and is involved in transportation of small hydrophobic molecules across the bacterial outer membrane (Hong et al. 2006, Touw et al. 2010).

The OmpW of *Vibrio alginolyticus* has been described as a 214 amino acid protein with a predicted molecular weight of 23.3 kDa (Qian et al. 2007), and its gene found on chromosome II. It is likely to play an important role in bacterial physiology, contributing to the survival of *V. alginolyticus* within the host as well as in the wider environment (Nandi et al. 2005). OmpW is a highly conserved membrane protein in *Vibrio* species, and polyclonal antibody raised against recombinant OmpW from *V. parahaemolyticus* recognized the OmpW homologues from other strains of *Vibrio* species in immunoblotting studies (Qian et al. 2007). Therefore, OmpW could be a shared antigen among *Vibrio* species (Ma et al. 2011). Several reports have shown that vaccines consisting of OMPs can induce high protective immunity (Zhang et al. 2007, Cai et al. 2010). Therefore, OMPs are particularly attractive as vaccine candidates.

In the present study, we cloned the *ompW* gene from *Vibrio alginolyticus* and constructed a recombinant plasmid, pcDNA-*ompW*. This was used to immunize crimson snapper to explore the potential application of the *ompW* gene as a DNA vaccine candidate.

MATERIALS AND METHODS

Extraction of genomic DNA

Vibrio alginolyticus strain HY9901 was isolated from diseased mari-cultured crimson snapper *Lutjanus erythropterus* (Bloch) in Zhanjiang, China. The isolate was preserved in tryptone soya broth (Huangkai) supplemented with 2% NaCl and 20% glycerol at -80°C . The culture was retrieved on tryptone soya agar (Huangkai) plates supplemented with 2% NaCl and incubated at 28°C . Genomic DNA was extracted using the MiniBEST Bacterial Genomic DNA Extraction Kit (Takara).

Expression and purification of the recombinant OmpW

The primers were designed based on the *ompW* gene sequence of *Vibrio alginolyticus* cloned by our laboratory (GenBank accession no. GQ891116). The sense primer, *ompW*-F1, was 5'-CGC CAT ATG AAA AAA ACA ATC TGC AGT CTA GC-3', corresponding to the *ompW* sequence from nucleotide 1 (the coding sequence starts at nucleotide 1) and containing an *Nde*I restriction site (underlined). The anti-sense primer, *ompW*-R1, was 5'-CCG CTC GAG GAA CTT GTA ACC GCC GCT GAT-3', corresponding to the *ompW* sequence from nucleotides 621 to 642 (the coding sequence ends at nucleotide 645) and containing an *Xho*I restriction site. The stop codon TAA was deleted in the *ompW*-R1 primer. Genomic DNA from *V. alginolyticus* strain HY9901 was used as the template in PCR. The PCR was performed in 25 μl of reaction mixture which contained 1 $\times$ standard PCR reaction buffer (Takara), 50 ng of genomic DNA, 100 pmol l^{-1} of each primer, 200 $\mu\text{mol l}^{-1}$ of each deoxynucleoside triphosphate (Takara) and 1.5 U of Ex *Taq* DNA polymerase (Takara). The amplification was carried out as follows: 5 min at 94°C ; 30 cycles of 45 s at 94°C , 45 s at 58°C , 90 s at 72°C ; then 10 min at 72°C for further extension. The PCR product was cloned into the pMD18-T plasmid (Takara) and then sequenced by Shanghai Sangon Biological Engineering Technology and Services (SSBETS). The sequence was used to perform a BLAST search in GenBank, then multiple alignment and similarity analysis was carried out using the CLUSTAL X program (Thompson et al. 1997).

To construct an expression vector for *ompW*, the purified PCR product and the expression vector pET21a (Novagen) were digested with *Nde*I and

XhoI and ligated together. pET21a-*ompW* was constructed by inserting the DNA fragment containing the *ompW* gene open reading frame without a stop codon into the 5'-*NdeI* and 3'-*XhoI* sites of pET21a in-frame with the 6-histidine (His) tag. The 6-His-tagged protein can be purified using a Ni²⁺-iminodiacetic acid (IDA) Sepharose affinity column. The promoter and coding region of the recombinant pET21a-*ompW* vector was confirmed by DNA sequencing and the vector was transformed into *E. coli* BL21 (DE3) for expression. The *E. coli* BL21 (DE3) cells containing pET21a-*ompW* were cultivated and induced with 1 mM isopropyl-thio- β -D-galactoside (IPTG) in Luria-Bertani broth (LB; Huangkai) containing 50 mg ml⁻¹ kanamycin at 37°C. Then 100 ml of the induced bacterial culture was centrifuged at 12 000 $\times g$ for 10 min at 4°C and the pellet was re-suspended in 15 ml of pH 7.4 lysis buffer (20 mM phosphate-buffered saline [PBS], 0.5 M NaCl and 8 M urea). The cell suspension was sonicated and the lysed mixture was centrifuged at 12 000 $\times g$ for 10 min at 4°C. Then the supernatant was collected and filtered through a 0.22 μ m spin filter (Ultrafree-MC; Millipore). The filtered supernatant was loaded into a Ni²⁺-IDA Sepharose affinity column. The recombinant OmpW was purified according to the manufacturer's instructions and analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Construction and preparation of DNA vaccines

Primers (*ompW*-F2: 5'-CGG AAT TCA TGA AAC AGG GTG ACT TCG TTC TTC GT-3'; *ompW*-R2: 5'-CCG CTC GAG CTA CTT GTA ACC GCC GCT GAT-3') containing specific restriction sites (*EcoRI* / *XhoI*, underlined) were designed to amplify the *ompW* gene for cloning into a eukaryotic expression vector. Genomic DNA from *Vibrio alginolyticus* strain HY9901 was used as the template in PCR. The PCR reaction mixtures were comprised as described above. The amplification was carried out as follows: 5 min at 94°C; 30 cycles of 45 s at 94°C, 45 s at 63°C, 60 s at 72°C; then 10 min at 72°C for further extension. The PCR product was purified using the QIAquick-spin PCR Purification Kit (Qiagen), cloned into the eukaryotic expression vector pcDNA3.1 (Invitrogen), and transformed into *E. coli* DH5 α grown in LB media at 37°C. Recombinant clones were selected by ampicillin resistance and confirmed

by restriction analysis of recombinant plasmids, and then sequenced at SSBETS. pcDNA-*ompW* was prepared on a large scale in *E. coli* DH5 α grown in LB media at 37°C and purified using PureLink™ HiPure Plasmid Filter Purification Kits (Invitrogen). The purified pcDNA-*ompW* plasmid was suspended in PBS at a final concentration of 0.1 mg ml⁻¹ and stored at -20°C until use.

Preparation of antiserum against the pcDNA-*ompW* vaccine and western blot analysis

Antiserum against the pcDNA-*ompW* was raised in New Zealand white rabbits *Oryctolagus cuniculus* by means of subcutaneous injection of 0.1 ml pcDNA-*ompW* (0.1 mg ml⁻¹). All rabbits were boosted 4 wk later with the same antigen preparations. The rabbits were bled 4 wk after the booster injection, and the blood was clotted at room temperature for 1 h and then stored at 4°C overnight. The separated antiserum obtained by centrifugation was stored at -70°C until use. Crude OMPs of *Vibrio alginolyticus* strain HY9901 were prepared according to Cai et al. (2010). The crude OMPs, recombinant OmpW, total protein of induced recombinant *E. coli* BL21 (DE3) harbouring pET21a-*ompW*, total protein of non-induced recombinant *E. coli* BL21 (DE3) harbouring pET21a-*ompW* and the total protein of induced *E. coli* BL21 (DE3) were electrophoresed by SDS-PAGE on a 5% stacking gel and 15% separating gel under denaturing conditions. The proteins were electrically transferred from the gel to nitrocellulose paper using a semi-dry apparatus (Bio-Rad). The sera from rabbits vaccinated with pcDNA-*ompW* were used as primary antibody at a 1:100 dilution. Non-immunized rabbit sera were used as primary antibody in the negative control. The secondary antibody was peroxidase-conjugated goat-anti-rabbit IgG (Sigma) used at a 1:5000 dilution. The antibody-bound proteins were then visualized by 3,3'-diaminobenzidine (DAB).

Vaccination

Healthy crimson snapper confirmed free of disease and weighing approximately 50 g were divided into 3 groups (100 fish in each group) for vaccination. Every group was kept in a 1000 l tank with fresh running water at 25°C and fed to satiation daily with commercial dry pellets. The fish were allowed to acclimatise for 14 d before vaccination. In the test

group, fish were injected intramuscularly with 0.1 ml (0.1 mg ml^{-1}) pcDNA-*ompW*; in control group I, fish were injected intramuscularly with 0.1 ml (0.1 mg ml^{-1}) pcDNA3.1; in control group II, fish were injected intramuscularly with 0.1 ml PBS.

Detecting expression of the *ompW* gene by RT-PCR

Total RNA was extracted from individual tissues including gill, head kidney, heart, liver, spleen and injection site muscle from 6 fish from each group, at 7 and 28 d post-vaccination with Trizol reagent (Invitrogen). Genomic DNA was removed using the DNA-freeTM DNase Treatment and Removal Kit (Ambion). RNA concentration was measured with a spectrophotometer (NanoDrop Technologies). RNA was reverse transcribed into cDNA and amplified using the OneStep RT-PCR Kit (Qiagen) according to the manufacturer's instructions and using the primers *ompW*-F3 (5'-CAC CAA GTG TTT TCG CTC AT-3') and *ompW*-R3 (5'-CTG CTT TGT AAG TTG CTT CTG T-3'). RT-PCR was performed with a Bio-Rad C1000 thermal cycler using the following thermocycling conditions for the PCR reaction: denaturation for 5 min at 95°C, 30 amplification cycles of 30 s at 95°C, 30 s at 58°C, 30 s at 72°C; then 10 min extension at 72°C. The amplified fragments were sequenced by SSBETS.

Analysis of specific antibody levels

During 10 wk post-vaccination, 6 fish from each treatment group were assayed weekly for *OmpW*-specific antibodies in serum by enzyme-linked immunosorbent assay (ELISA). For this, 96-well plates were coated using 100 μl ($10 \mu\text{g ml}^{-1}$) of the purified recombinant *OmpW*. Fish serum samples in 2-fold serial dilutions (2^0 to 2^{16}) were added to the microplate, which had been blocked with 2% BSA. Antibody binding to the antigen was detected using rabbit anti-crimson snapper IgM antibodies at a 1:500 dilution. Preparation of rabbit anti-crimson snapper IgM antibodies was carried out according to Cai et al. (2013). Plates were incubated with horse-radish peroxidase-conjugated goat anti-rabbit IgG at a 1:1000 dilution. The reaction was developed with the 3,3',5,5'-tetramethylbenzidine substrate and H_2O_2 , and stopped with 2 M H_2SO_4 . Optical density was measured at 450 nm using a microplate reader (μQuant ; BioTek).

Bacterial challenge experiments

At 49 d post-immunization, 69, 72 and 72 fish of the vaccinated, control I and control II groups, respectively, were anaesthetized and challenged by intramuscular injection with 0.1 ml bacterial suspension (1×10^7 colony-forming units ml^{-1}) of *Vibrio alginolyticus* strain HY9901 (Kumar et al. 2007). Fish that died during the course of the challenge were autopsied to determine the cause of death and to detect the presence of *V. alginolyticus* in the tissues by bacterial culture in thiosulphate citrate bile salts-sucrose agar. Relative percentage survival (RPS) was calculated from the cumulative mortalities (M_{vac} in vaccinated fish; M_{unvac} in unvaccinated control fish) using:

$$\text{RPS} = [1 - (M_{\text{vac}}/M_{\text{unvac}})] \times 100 \%$$

Statistical analysis

Data are expressed as mean $\pm$ SD. A statistical analysis was performed to assess serum antibody levels and mortality for significance ($p < 0.05$) using 1-way ANOVA (Kumar et al. 2007). Probabilities of 0.05 or less were considered statistically significant.

RESULTS

Cloning, expression and purification of recombinant *OmpW*

A 657 bp fragment was obtained from genomic DNA of *Vibrio alginolyticus* strain HY9901 by PCR amplification. The fragment contained an open reading frame (ORF) of 645 nucleotides which encodes 214 amino acids with a deduced molecular mass of 23.47 kDa. The nucleic acid sequence similarities with *ompW* of *Vibrio* sp. Ex25 (GenBank accession no. CP001806), *V. parahaemolyticus* RIMD 2210633 (GenBank accession no. BA000032) and *Photobacterium damsela* (GenBank accession no. DQ251175) were 96, 92 and 92%, respectively. The amino acid sequence similarities with the proteins of *Vibrio* sp. Ex25, *V. parahaemolyticus* RIMD 2210633 and *V. harveyi* 1DA3 were 96, 92, and 91%, respectively.

Sequencing revealed that the *ompW* gene was correctly cloned into the expression vector pET21a. The *ompW* gene was efficiently expressed following induction with IPTG and the recombinant protein was in inclusion body form. A novel protein band corresponding to 24 kDa was detected in *E. coli* BL21 (DE3) harbouring the recombinant pET21a-*ompW*.

vector by SDS-PAGE (Fig. 1). The molecular weight of the recombinant protein was identical with that predicted from the nucleotide sequence of the *ompW* gene. After His-tag affinity chromatography, the expressed proteins were purified to a single band of the expected size, as confirmed by SDS-PAGE.

Western blot analysis

Following western blot analysis, a protein band was only recognized in the crude OMPs, purified recombinant OmpW and total protein of induced recombinant *E. coli* BL21 (DE3) harbouring pET21a-*ompW* (Fig. 2A, Lanes 1, 2 and 3). No band was found when western blotting was performed with the non-immunized rabbit serum (Fig. 2B).

Analysis of *ompW* gene expression

RT-PCR was used to detect the distribution of the pcDNA-*ompW* vector and/or expression of the *ompW* gene in different tissues of crimson snapper. The fragment, expected to be 525 bp in length, was amplified from cDNA synthesized from the total RNA extracted from gill, head kidney, heart, liver, spleen and injection site muscle of fish vaccinated with pcDNA-*ompW* on 7 and 28 d post-vaccination (Fig. 3). There was no band from the negative control groups I and II. Sequence analysis confirmed that the 525 bp fragment was the partial *ompW* gene.

Fig. 1. SDS-PAGE analysis of the expression products of pET21a-*ompW* in *E. coli* BL21 (DE3). Lane M, protein marker; Lanes 1 to 4, total protein of recombinant *E. coli* BL21 (DE3) harbouring (Lane 1) pET21a, not induced, (Lane 2) pET21a, induced, (Lane 3) pET21a-*ompW*, not induced, and (Lane 4) pET21a-*ompW*, induced; Lane 5, purified recombinant OmpW

Fig. 2. Western blot analysis with (A) serum of rabbits immunized with pcDNA-*ompW* and (B) non-immunized rabbit serum. Lane M, protein marker; Lane 1, crude outer membrane proteins of *Vibrio alginolyticus* strain HY9901; Lane 2, purified recombinant OmpW; Lanes 3 and 4, total protein of (Lane 3) induced and (Lane 4) non-induced recombinant *E. coli* BL21 (DE3) harbouring pET21a-*ompW*; Lane 5, total protein of induced *E. coli* BL21 (DE3)

Fig. 3. *Lutjanus erythropterus*. Expression of the *ompW* gene in various fish tissues at (A) 7 and (B) 28 d post-vaccination by RT-PCR. Lane M, DL2000 marker; Lane 1, gill; Lane 2, head kidney; Lane 3, heart; Lane 4, liver; Lane 5, spleen; Lane 6, injection site muscle; Lane 7, injection site muscle of fish in negative control group I; Lane 8, injection site muscle of fish in negative control group II

Analysis of serum antibody levels

The level of OmpW-specific serum antibodies in crimson snapper is shown in Fig. 4. The results indicated that an OmpW-specific antibody titre was detected in all sera of the vaccinated fish. During the 1st to the 10th week post-vaccination, $\log_2$ antibody titres in the sera of vaccinated groups all reached above 5.14, and the maximum reached 13.31, while that of control group I injected with the empty vector pcDNA3.1 was only 1.76 to 4.31 at all times, and that of control group II injected with PBS was 1.21 to

1.97 at all times. Comparison of the means of $\log_2$ antibody titres between vaccinated and control groups revealed a statistically significant difference ($p < 0.05$).

Efficacy of the pcDNA-ompW vaccine

The cumulative mortality of crimson snapper after challenge with virulent *Vibrio alginolyticus* strain HY9901 is shown in Fig. 5. The fish immunized with the pcDNA-ompW vaccine displayed a high level of protection. The fish mortality rate was significantly lower ($p < 0.01$) in the vaccinated groups than in the control groups after challenge with virulent *V. alginolyticus* strain HY9901. The fish in the control group began to die 2 d post-challenge, with a sudden increase in the death rate between 3 and 8 d, followed by a decrease until 14 d post-challenge. The dead fish in the control and vaccinated groups showed typical signs of vibriosis including anorexia, skin ulcers and necrosis of the tail fin and scales and no pathogen other than *V. alginolyticus* was isolated from dead fish. The RPS value for the pcDNA-ompW vaccine was 92.53%.

Fig. 4. *Lutjanus erythropterus*. Analysis of the OmpW-specific serum antibody titres. Vaccinated group, fish vaccinated with pcDNA-ompW; control group I, fish injected with the empty vector pcDNA3.1; control group II, fish injected with PBS. Each bar represents mean $\log_2$ value $\pm$ SD of the highest dilution of the serum when $P/N \geq 2.1$, where P = optical density (OD_{492}) of the inspected serum, N = OD_{492} of the negative serum. * $0.01 < p < 0.05$, ** $p < 0.01$

Fig. 5. *Lutjanus erythropterus* challenged with *Vibrio alginolyticus* strain HY9901. Cumulative mortality curves. (◆) Test fish vaccinated with pcDNA-ompW, (■) control fish injected with the empty vector pcDNA3.1, (▲) control fish injected with PBS

DISCUSSION

The outer membrane of Gram-negative pathogenic bacteria has an important role in adherence to host cells, uptake of nutrients from the host and subverting host defence mechanisms. The OMPs of these bacteria have been reported to play an important role during bacterial infection and induction of host immune response, and they are often able to induce neutralization antibodies that are bactericidal (Gatto et al. 2002), inducing cell-mediated immunity and inhibiting bacterial colonization in hosts (Sengupta et al. 1992). OMPs have shown even higher immunogenicity than inactivated whole cells, probably because chemicals such as formalin, used in whole cell prepara-

tions, damage the structure of the bacterium's antigens (Prado et al. 2005). Some of the important attributes of a candidate molecule for vaccine development are that the candidate should be highly conserved among various members of the same genus, should be expressed on the surface of pathogens so that antigen-presenting cells can easily recognize them, and should be immunogenic (Karlsen et al. 2011). The BLAST results showed that the sequence of *ompW* is well conserved among different *Vibrio* species. This is one of the important reasons that OMPs are particularly attractive for use as vaccine candidates. When rabbit polyclonal antibodies against the product of the DNA vaccine containing the *ompW* gene were tested in western blots with crude OMPs and recombinant OmpW, a strong reaction was observed against both. A putative signal peptide sequence of 21 residues at the N-terminus of the OmpW protein has been predicted through analysis of the sequence of the *ompW* gene. As a secreted protein, mature OmpW is released and the signal peptide cleaved off after translocation (Movva et al. 1980, Jalajakumari & Manning 1990). This explains the lower molecular weight of the mature OmpW in the crude OMPs compared to that of the recombinant OmpW (Fig. 2, Lanes 1, 2 and 3). The antibody reaction to both crude OMPs and recombinant OmpW proves that the DNA vaccine containing the *ompW* gene of *V. alginolyticus* retained natural antigenicity. Therefore, this supports the use of the *ompW* gene as a potential DNA vaccine candidate. Several bacterial *Omp* genes have been reported as being potential DNA vaccine candidates and can induce effective protection of the host, including those of *V. harveyi* (Wang et al. 2011), *Salmonella typhimurium* (Jang et al. 2011), *Edwardsiella tarda* (Sun et al. 2011) and *Mannheimia haemolytica* (Ayalew et al. 2010). In the present study, we demonstrated that an intramuscularly injected DNA vaccine containing the gene encoding for *V. alginolyticus* OmpW conferred significant protection to vaccinated fish challenged with *V. alginolyticus*.

Little is yet known about the tissue distribution, degradation and persistence of plasmid DNA in fish following DNA vaccination. Several reports have shown that DNA vaccines are able to reach other organs, such as kidney, spleen and gill, some days post-immunization, indicating that plasmid DNA can avoid local degradation at the administration site and in the blood plasma (Ayalew et al. 2010, Liang et al. 2011). In our study, although the PCR was performed using the *ompW*-F3 and *ompW*-R3 primers and the RNA template was treated with DNA-freeTM DNase,

it is still possible that pcDNA-*ompW* vector contamination remained in the total RNA extracted from the tissues, as negative controls were not run for the reverse transcription step. The RT-PCR results therefore show that the pcDNA-*ompW* was distributed and/or expressed in several fish tissues including gill, head kidney, heart, liver, spleen and injection site muscle on 7 and 28 d post-vaccination. These results are in close agreement with those obtained by Liang et al. (2011).

We assessed the antibody levels generated against *Vibrio alginolyticus* OmpW in vaccinated and unvaccinated crimson snapper by ELISA. The results show that immunization of crimson snapper with the DNA vaccine containing the *ompW* gene induced significant protection against experimental challenge with *V. alginolyticus*. The DNA vaccine induced a significant antibody immune response in fish against the OmpW protein. The crimson snapper in control group I injected with pcDNA3.1 produced a higher serum antibody titre against the OmpW recombinant protein than those in control group II injected with PBS. This was possibly due to the polyclonal antibody characteristics of the fish serum used as the primary antibody source in the ELISA experiments. Comparison of the means of log₂ antibody titres between the 2 control groups revealed their differences in titre were not statistically significant ($p > 0.05$). In this study, crimson snapper vaccinated with pcDNA-*ompW* were protected against *V. alginolyticus* with an RPS of 92.53%. However, a DNA vaccine encoding the *ompU* gene of *V. harveyi* provided protection in *Scophthalmus maximus* with an RPS of 51.5% (Wang et al. 2011). The difference in RPS reported for the 2 DNA vaccines may have been caused by differences in DNA sequence, bacterial virulence and immune response in the tested animals. Furthermore, other differences, including vaccine dose, the time which elapsed between vaccination and challenge, bacterial dose and inoculation method in the challenge experiment, and the fish species, could have contributed to the observed differences in the efficacy of the vaccines.

The DNA vaccine against infectious *Vibrio alginolyticus* is effective at protecting crimson snapper, but intramuscular injection is required and this makes the vaccine difficult to apply in the crimson snapper farming industry. More natural routes of delivery should be studied, such as oral administration or immersion, in order to determine efficient delivery methods for DNA vaccines in aquaculture situations.

In conclusion, the *ompW* gene from *Vibrio alginolyticus* was cloned and expressed in *E. coli*, and proved to be a good candidate for a vaccine target against *V. alginolyticus* in crimson snapper. The DNA vaccine containing the *ompW* gene offers promise for the development of a protective vaccine for piscine vibriosis.

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