Chinese Journal of Oceanology and Limnology
Vol. 34 No. 6, P. 1247-1257, 2016
http://dx.doi.org/10.1007/s00343-016-5096-z

Immunogenicity and protective role of antigenic regions from five outer membrane proteins of *Flavobacterium columnare* in grass carp *Ctenopharyngodon idella**

LUO Zhang (罗璋)[1, 2, 3], LIU Zhixin (刘志新)[2], FU Jianping (付建平)[2], ZHANG Qiusheng (张秋胜)[2], HUANG Bei (黄贝)[4], NIE Pin (聂品)[2, **]

[1] *College of Fisheries, Huazhong Agricultural University, Wuhan 430070, China*
[2] *State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China*
[3] *Tianjin Fishery Research Institute, Tianjin 300221, China*
[4] *College of Fisheries, Jimei University, Xiamen 361021, China*

Received Mar. 28, 2015; accepted in principle Jun. 8, 2015; accepted for publication Aug. 25, 2015
© Chinese Society for Oceanology and Limnology, Science Press, and Springer-Verlag Berlin Heidelberg 2016

Abstract *Flavobacterium columnare* causes columnaris disease in freshwater fish. In the present study, the antigenic regions of five outer membrane proteins (OMPs), including zinc metalloprotease, prolyl oligopeptidase, thermolysin, collagenase and chondroitin AC lyase, were bioinformatically analyzed, fused together, and then expressed as a recombinant fusion protein in *Escherichia coli*. The expressed protein of 95.6 kDa, as estimated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, was consistent with the molecular weight deduced from the amino acid sequence. The purified recombinant protein was used to vaccinate the grass carp, *Ctenopharyngodon idella*. Following vaccination of the fish their IgM antibody levels were examined, as was the expression of *IgM*, *IgD* and *IgZ* immunoglobulin genes and other genes such as *MHC Iα* and *MHC IIβ*, which are also involved in adaptive immunity. Interleukin genes (*IL*), including *IL-1β*, *IL-8* and *IL-10*, and type I and type II interferon (*IFN*) genes were also examined. At 3 and 4 weeks post-vaccination (wpv), significant increases in *IgM* antibody levels were observed in the fish vaccinated with the recombinant fusion protein, and an increase in the expression levels of *IgM*, *IgD* and *IgZ* genes was also detected following the vaccinations, thus indicating that an adaptive immune response was induced by the vaccinations. Early increases in the expression levels of *IL* and *IFN* genes were also observed in the vaccinated fish. At four wpv, the fish were challenged with *F. columnare*, and the vaccinated fish showed a good level of protection against this pathogen, with 39% relative percent survival (RPS) compared with the control group. It can be concluded, therefore, that the five OMPs, in the form of a recombinant fusion protein vaccine, induced an immune response in fish and protection against *F. columnare*.

Keyword: *Flavobacterium columnare*; outer membrane protein; antigen; immunogenicity; vaccine; immune response; grass carp

1 INTRODUCTION

Flavobacterium columnare, a Gram-negative bacterium, is the causative agent of columnaris disease of freshwater fish in worldwide aquaculture (Decostere et al., 1999; Liu et al., 2008). A wide range of fish species can become infected with this bacterium, including cold-water fish such as the rainbow trout, *Oncorhynchus mykiss* (Barnes et al., 2009), tropical fish such as the Nile tilapia, *Oreochromis niloticus* (Figueiredo et al., 2005), and warm-water fish, as such the channel catfish, *Ictalurus punctatus* (Darwish and Mitchell, 2009) and the grass

* Supported by the National Basic Research Program of China (973 Program) (No. 2009CB118703), the Science and Technology Program of Xiamen Southern Oceanographic Center (No. 14PYY050SF03), and the National Science and Technology Support Program Project of China (No. 2012BAD25B02)
** Corresponding author: pinnie@ihb.ac.cn

carp, *Ctenopharyngodon idella* (Liu et al., 2012). Columnaris disease often causes high mortality resulting in severe economic losses in the fish farming industry (Liu et al., 2012). Over the last two or three decades, research on *F. columnare* as a bacterial pathogen has been related mainly to its diagnosis, epidemiology, pathology and preventive measures against infection with it (see Declercq et al., 2013 for a review). In contrast, identification of potential virulence factors in this bacterium has attracted the attention of fewer studies (Xie et al., 2004; Li et al., 2010), while the development of a vaccine against columnaris disease has been the focus of some research over the last a few years. Using formalin-killed bacteria, Leal et al. (2010) found that intraperitoneally and intramuscularly injected bacteria provoked a specific antibody response in Nile tilapia fingerlings, but a protective effect was not observed in challenge infections. Shoemaker et al. (2011) developed an attenuated live vaccine by passing the bacterium through rifampicin-supplemented culture, and found that this vaccine was effective at protecting channel catfish and largemouth bass (*Micropterus salmoides*) from *F. columnare* infections. Meanwhile, Olivares-Fuster et al. (2010) tried to develop a recombinant vaccine, and observed a specific antibody response in channel catfish injected with the recombinant protein of a heat shock protein encoded by *dnaJ* of *F. columnare* although no protection was detected in the immunized fish when challenged with *F. columnare*. However, vaccination trials of other recombinant proteins based on *F. columnare* proteins are not available in the scientific literature in relation to the development of possible vaccines against columnaris disease.

In our previous study, five proteins, including a zinc metalloprotease, a prolyl oligopeptidase, thermolysin, collagenase and chondroitin AC lyase, which are all components of outer-membrane proteins (OMPs) in *F. columnare*, were screened by employing an immune-related screening method (Xie et al., 2004). As these proteins were immune-reactive with host sera, it is hypothesized that they could be used as possible immunogens for the development of recombinant protein vaccines. In the present study, the antigen regions of the five proteins were bioinformatically analyzed, and all such regions in these proteins were cloned into a pET-32a vector to express them as a single fusion protein. The potential immune protection of this fusion protein was then examined in the grass carp, *C. idella*, a freshwater fish species cultured widely and one that suffers severely from columnaris disease in China (Wang et al., 2010). The immune response, at the mRNA level, was also examined in the immunized fish in terms of the expression of *IgM*, *IgD* and *IgZ* genes, and *MHC Iα* and *MHC IIβ* genes, as well as some innate immunity genes including type I and type II interferon (*IFN*) genes and interleukin (*IL*) genes.

2 MATERIAL AND METHOD

2.1 Bacterial strains and culture conditions

The G_4 strain of *F. columnare*, isolated originally from the outbreak of gill-rot disease in grass carp in 1972 (Lu et al., 1975), was used in the present study. It has been continuously employed in our research and was cultivated at 25°C with gentle agitation in Shieh broth as recommended by Decostere et al. (1997). *Escherichia coli* TOP10 and DE3 strains (TaKaRa, Dalian, China), used for gene cloning and expression studies, respectively, were cultivated at 37°C in Luria-Bertani broth.

2.2 Amplification and fusion of the antigenic regions of the OMP genes

The sequences coding for zinc metalloprotease (GenBank Accession No. AY387596), prolyl oligopeptidase (AY387598), thermolysin (EF140791), collagenase (EF501979) and chondroitin AC lyase (AY912281) were retrieved from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The TMHMM Server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/) and DNAStar software were used to evaluate transmembrane, hydrophilic and antigenic regions in the aforementioned proteins. Based on the results, five pairs of primers with appropriate restriction endonuclease sites were designed to amplify the antigenic regions of the five OMP genes. All primers used in this study are listed in Table 1. Genomic DNA prepared from *F. columnare* was used as the PCR template. Reactions were run in a PTC-100 thermocycler (MJ Research, USA) by heating the tubes at 94°C for 4 min followed by 35 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, and then a final extension at 72°C for 10 min. After restriction enzyme digestion, the PCR products were ligated with T4 DNA ligase. Subsequently, the ligated product was used as the PCR template for amplifying the series of ligated OMP gene segments using the Maz-f

Table 1 PCR primers used in this study

Gene name	Primer	Sequence (5′→3′)	Application
Zinc metalloprotease	Maz-f	CGGCCATGGTCCAAATTGGTTTCAAAAATGGTG (*Nco*I)	Construction of chimeric antigen
	Maz-r	CGGGTCGACGTAAGCACCTGTTTTAGGAGATAAT (*Sal*I)	
Prolyl oligopeptidase	Pop-f	CGCGTCGACGAACCTTACGATGCTCCACAAAAAC (*Sal*I)	
	Pop-r	CGGCTTAAGGACTTTTTTACCATCTTTACTCCAT (*Bsp*TI)	
Thermolysin	Thermo-f	CGCCTTAAGTTAAGCGGATCATCCTATATATTAT (*Bsp*TI)	
	Thermo-r	CGGCATATGAAAATAAGTACCACTACCATCGCCA (*Nde*I)	
Collagenase	Collage-f	CGCCATATGGAGATGCGAAATGTGTATGAAC (*Nde*I)	
	Collage-r	CGGTCGCGATTTTTGATCCATGTTTTCACGATGG (*Bsp*681)	
Chondroitin AC lyase	ClsA-f	CGCTCGCGAAAATCCGCATTACAAGATGCCCATG (*Bsp*681)	
	ClsA-r	CGCGAGCTCCCTGATGCTCCATTTTTCCCATCAG (*Sac*I)	
β-actin	β-actin-f	AGAGTATTTACGCTCAGGTGGG	Quantitative real-time PCR
	β-actin-r	CCTTCTTGGGTATGGAGTCTTG	
IgM	IgM-f	TGGCAAATACAATAAACACC	
	IgM-r	GGACTATAACAGCAGCAACA	
IgD	IgD-f	GTGGCAACTAAATGGGAC	
	IgD-r	TAAATGGACTTTGGGATGA	
IgZ	IgZ-f	TGAATAATGACCGGGTGG	
	IgZ-r	GGATTTCCTGCTTGATGTG	
IL-1β	IL-1β-f	GGAGAATGTGATCGAAGAGCGT	
	IL-1β-r	GCTGATAAACCATCCGGGA	
IL-8	IL-8-f	AGGTCTGGGTGTAGATCCACGCTG	
	IL-8-r	TTAGTGTGAAAACTCACATGATCTCT	
IL-10	IL-10-f	ACTGACTGTTGCTCATTTGTGG	
	IL-10-r	TACTGCTCGATGTACTTAAAGAG	
MHC Iα	MHC Iα-f	CCTGGCAGAAAAATGGACAAG	
	MHC Iα-r	CCAACAACACCAATGACAATC	
MHC IIβ	MHC IIβ-f	TACTACCAGATTCACTCGG	
	MHC IIβ-r	CGGGTTCCAGTCAAAGAT	
IFN	IFN-f	GGTGAAGTTTCTTGCCCTGACCTTAG	
	IFN-r	CCTTATGTGATGGCTGGTATCGGG	
IFN-γ	IFN-γ-f	AGAAACCCTATGGGCGATTA	
	IFN-γ-r	CTGCCTCTTGATGCTTTTGG	

In the primer sequences, the underlined nucleotides represent restriction enzyme sites with the specific enzymes indicated in parentheses. f: forward primer; r: reverse primer.

and ClsA-r primer pair designed for this purpose. The PCR program used to amplify the gene segments was as follows: 5 min at 94°C, 40 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C and a 10 min final extension at 72°C. The resultant PCR product was inserted into the pMD18T vector (Tiangen, Beijing, China) and then transformed into *E. coli* TOP10 competent cells. The recombinant transformant was confirmed by DNA sequencing.

2.3 Expression and purification of recombinant protein

Plasmids extracted from the antigen-expressing *E. coli* TOP10 clone were digested with *Nco*I and *Sac*I restriction enzymes, and the digested fragment including the target fragment was inserted into a pET-32a vector (Novagen, Germany). The sequenced plasmid was transformed into *E. coli* DE3 cells and

the recombinant strain was induced with 0.5 mmol/L isopropyl β-d-1-thiogalactopyranoside (IPTG). After 6 h induction, the bacterial cells were harvested, resuspended in phosphate-buffered saline (PBS; pH, 7.4), and lysed by sonication on ice. Inclusion bodies were solubilized in denaturing solution and purified using Ni-NTA based affinity chromatography as described by Poobalane et al. (2010). The purified protein was subsequently analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized after staining with Coomassie brilliant blue R-250. As a negative control, an induced culture of *E. coli* DE3 previously transformed with the empty pET-32a vector was also subjected to purification using the same procedure.

2.4 Fish vaccinations

The purified recombinant protein was quantified by the bicinchoninic acid method of Smith et al. (1985), and its concentration was adjusted to 0.5 mg/mL. Grass carp, weighing 80–100 g each, purchased from the experimental station of the College of Fisheries, Huazhong Agricultural University, China, were acclimatized at 25°C in our laboratory for three weeks before commencing the experiments. One hundred and twenty fish were divided randomly into two groups: individual fish in group 1 were each injected intraperitoneally with 1 μg recombinant protein/g body weight, and those in group 2 were each injected intraperitoneally with 1 μg of the pET-32a vector tag protein as a control group.

2.5 Preparation of a monoclonal antibody (mAb) against grass carp IgM

The recombinant IgM heavy chain constant region from grass carp was expressed and purified as reported by Yan et al. (2012) and Liu et al. (2012). The primers used were as follows: F-5′-GGTACCGTCATC-GCTGAGGCATCGG-3′ and R-5′-AAGCTTTTAAC CGCTCTTCCACTCAGAATAACT-3′. pET-32a was induced to express a protein of about 31.2 kDa (Yan et al., 2012). Hybridomas-secreting the mAb against grass carp IgM were established by fusing SP2/0 with spleen cells from BALB/c mice immunized with the purified IgM protein. Immunization, fusion and cloning were performed according to the method described by Al-Harbi et al. (2000).

2.6 Western blot analysis of the recombinant protein

The recombinant protein was electrophoresed on a 5% stacking gel and a 10% separating gel in Tris-glycine buffer, and then electroblotted onto a nitrocellulose membrane (Invitrogen, USA). After blocking with 5% (w/v) nonfat milk in PBS containing 0.1% Tween 20 (PBST), the membrane was incubated overnight with the serum (1:100 diluted in PBST) collected from the grass carp vaccinated with the recombinant protein at 4°C. Afterwards, the mouse anti-grass-carp IgM monoclonal antibody (1:5 000 diluted in PBST) and goat anti-rat IgG conjugated with horse radish peroxidase (HRP; 1:5 000 diluted in PBST) were used as the second antibody and the bound antibody, respectively. Immunoreactive bands on the blot were detected using a SuperSignal West Pico Trial kit (Thermo, USA) and an ECL Western blot system (Bio-Rad, USA) according to manufacturers' instructions.

2.7 Analysis of the antibody response

At 1-week intervals, serum was collected from the vaccinated group and control group for a period of four weeks. On each occasion, three fish from each group were anaesthetized with ethyl carbamate, and blood samples were collected individually from the caudal vein with a syringe. The blood samples were allowed to clot overnight at 4°C. Serum from each blood sample was obtained by centrifugation (6 000×*g*, 10 min), and the IgM antibody level was determined by enzyme-linked immunosorbent assay (ELISA) as described by Wang et al. (2013). Briefly, 96-well plates were coated with the purified recombinant protein at 10 μg/mL. Fish serum (1:100 diluted in PBST) and mouse anti-grass-carp IgM monoclonal antibody (1:5 000 diluted in PBST) were used as the primary antibody and secondary antibodies, respectively. HRP-conjugated goat anti-rat IgG (1:5 000 diluted in PBST) was added to plates and the color was developed with 3, 3', 5, 5'-tetramethylbenzidine substrate for 30 min. The reaction was stopped with 2.0 mol/L H_2SO_4. Plates were read with a microplate reader (Thermo, USA) at 450 nm.

2.8 Real time PCR quantification of immune genes following vaccination

Fish were sampled as described above, and each blood sample was used separately to extract total RNA using Trizol (Invitrogen, USA). The RNA was then dissolved in diethylpyrocarbonate-treated water, and digested with DNase I (Fermentas) to eliminate genomic DNA, before being used for cDNA synthesis with the RevertAid™ First Strand cDNA Synthesis

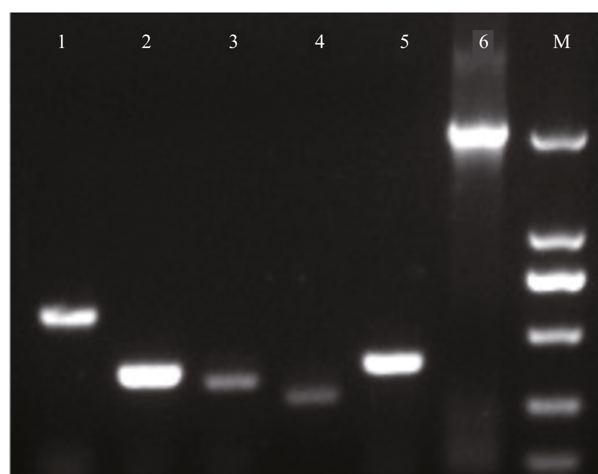

Fig.1 PCR amplification of the antigenic regions encoded by the DNA sequences of the five outer membrane protein (OMP) genes in *Flavobacterium columnare* and the fused DNA sequence of the antigenic regions

M indicates DL2000 markers at 2 000, 1 000, 750, 500, 250 and 100 bp; lane 1: antigen region sequence of zinc metalloprotease, 620 bp; lane 2: prolyl oligopeptidase, 393 bp; lane 3: thermolysin, 363 bp; lane 4: collagenase, 315 bp; lane 5: chondroitin AC lyase, 410 bp; lane 6: the full-length fused sequence containing the antigen regions of the five OMP genes, 2 053 bp.

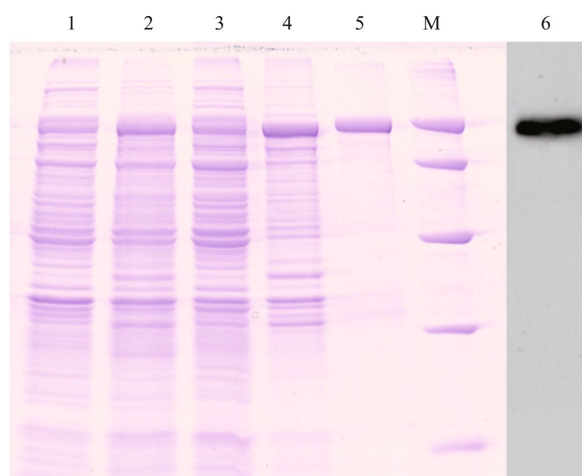

Fig.2 SDS-PAGE and Western blot analysis of the recombinant fusion protein

M indicates MK039 marker at 97.4, 66.2, 43.0, 31.0 and 20.1 kDa; lane 1: protein expression from recombinant plasmids in *E. coli* DE3 without IPTG; lane 2: protein expression from recombinant plasmids in *E. coli* DE3 with 0.5 mmol/L IPTG with a 6 h induction; lane 3: supernatant after sonication; lane 4: inclusion bodies; lane 5: purified recombinant fusion protein; lane 6: Western blot of the purified recombinant fusion protein.

Kit (Fermentas), which was used according to the manufacturer's instructions. Real time quantitative PCR was performed using the primers listed in Table 1. The 20 μL PCR reaction mixtures contained 1 μL of each primer (0.5 μmol/L), 2 μL template, 6 μL H_2O and 10 μL 2×iQ™ SYBR Green Supermix (Bio-Rad), on the Bio-Rad CFX96™ Real-Time System (Bio-Rad). The PCR conditions involved an initial denaturation cycle of 95°C for 3 min, 45 cycles of 95°C for 20 s, 55°C for 20 s, and 72°C for 30 s. Each sample was run in triplicate. Relative gene expression levels were determined by the comparative threshold cycle method ($2^{-\Delta\Delta Ct}$) with β-actin used as the reference gene (Zhang et al., 2012).

2.9 Bacterial challenge experiment

Twenty-eight days post-immunization, thirty grass carp in each group were challenged by intra-peritoneal inoculation of 0.2 mL of an *F. columnare* G_4 suspension at a concentration of 5×10^8 colony-forming units/mL. Mortality was recorded daily for two weeks following the challenge, and cumulative mortality was calculated. Dead fish were removed and their gills and livers were streaked onto Shieh agar plates to confirm the presence of *F. columnare*. Relative percentage survival (RPS) was calculated as reported by Zhang et al. (2007): RPS=(1−[mortality of vaccinated fish/mortality of control fish])×100%.

2.10 Statistical analysis

Data are expressed as the mean±S.D. and significant differences in the antibody titers and the transcript levels of immune genes were determined using the Student's *t*-test ($P<0.05$).

3 RESULT

3.1 Fusion of antigenic regions of five OMPs of *F. columnare*

Sequences encoding the antigenic regions of five the OMPs (i.e., zinc metalloprotease, prolyl oligopeptidase, thermolysin, collagenase, and chondroitin AC lyase) were successfully PCR amplified, and the appropriate restriction sites were introduced into the PCR products. The five PCR products were fused by ligation with T4 DNA ligase, and the fused product was amplified using Maz-f and ClsA-r primers (Fig.1).

3.2 Expression, purification and immunogenicity of the fusion protein

A novel protein band was detected by SDS-PAGE in induced *E. coli* containing the recombinant plasmid, while no protein band was found at the same position in uninduced *E. coli* containing the recombinant plasmid (Fig.2). The molecular mass of the recombinant protein revealed by SDS-PAGE was

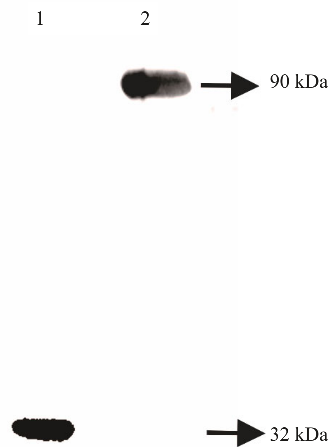

Fig.3 Western blot of the purified recombinant IgM heavy chain constant region and the IgM molecule in whole serum from grass carp using a mouse anti-grass carp IgM monoclonal antibody for detection

Lane 1: purified recombinant IgM heavy chain constant region with a mass of about 32 KDa, as reported by Yan et al. (2012); lane 2: IgM in whole serum from grass carp; the relative mobility is equivalent with that reported by Yan et al. (2012).

about 95.6 kDa, which is in agreement with the molecular weight deduced from its amino acid sequence. The recombinant protein reached an optimal expression level after 6 h induction with 0.5 mmol/L IPTG. After sonication of the cells, the recombinant protein was purified by Ni-NTA affinity chromatography, and the purity was >90%, as determined by bandscan software. The immunogenicity of the recombinant protein was analyzed by Western blotting with sera from the grass carp immunized with the recombinant protein followed by incubation with the mouse anti-grass carp IgM monoclonal antibody (Fig.3). The recombinant protein was recognized by the sera collected from the grass carp vaccinated with the recombinant protein (Fig.2), while the sera collected from the control group fish did not react with the recombinant protein (data not shown).

3.3 Analysis of the antibody response following fusion protein immunization

The IgM antibody levels in grass carp immunized with the recombinant fusion protein were measured with ELISA. As shown in Fig.4, the antibody titers in the immunized fish rose gradually from 1 to 4 weeks post-vaccination (wpv), and significantly higher levels were detected in the fish at 3 and 4 wpv (P<0.05).

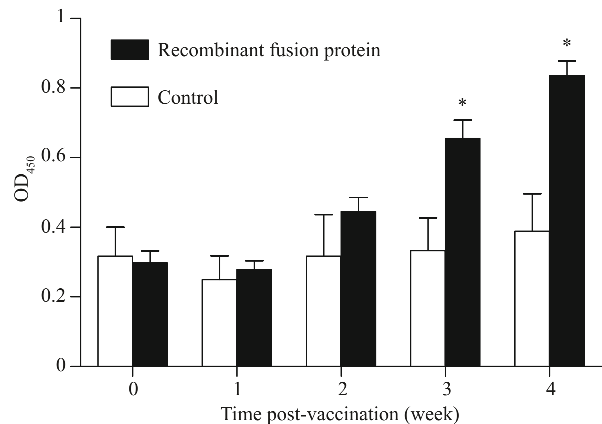

Fig.4 IgM antibody levels in grass carp vaccinated with the recombinant fusion protein as detected by using ELISA

Data are expressed as the mean±S.D. of OD_{450} values from the sera of three grass carp. Asterisks (*) indicate significant differences (P<0.05) among the different groups on the same sampling occasion.

3.4 Expression of immune genes following fusion protein immunization

The transcription profile of the immune genes was examined in the immunized fish; the relevant genes included *IgM*, *IgD* and *IgZ* immunoglobulin genes, two other genes, *MHC Iα* and *MHC IIβ*, which are also involved in the adaptive immune response, type I and type II IFNs, and *IL-1β*, *IL-8* and *IL-10* genes (Fig.5). Expression of *IgM*, *IgD* and *IgZ* immunoglobulin genes showed an increasing trend over the four week period following immunization (Fig.5a), and a significant increase was observed for all three Ig genes at 1 wpv, and for *IgM* at 3 wpv. The *IL* genes, *IL-1β*, *IL-8* and *IL-10*, showed significant increases at 1 wpv, but their levels declined gradually, although *IL-8* gene expression was significantly upregulated at 4 wpv (Fig.5b). Type I *IFN* and type II *IFN* expression was also detected, but only significantly at 1 wpv for type I IFN (Fig.5c). On other sampling occasions, *IFN* and *IFN-γ* expression levels were relatively low (Fig.5c). A decreased trend was also observed for *MHC Iα* and *MHC IIβ* expression, but *MHC Iα* was expressed at a significantly high level on 1 wpv and 3 wpv

3.5 Protection of grass carp from *F. columnare* challenge

At 4 weeks post-immunization, the grass carp were challenged with *F. columnare* G_4. In the group vaccinated with the recombinant fusion protein, the cumulative percent mortality was 56.7%, while in the control group it was 93.3% (RPS=39%; Fig.6). Death

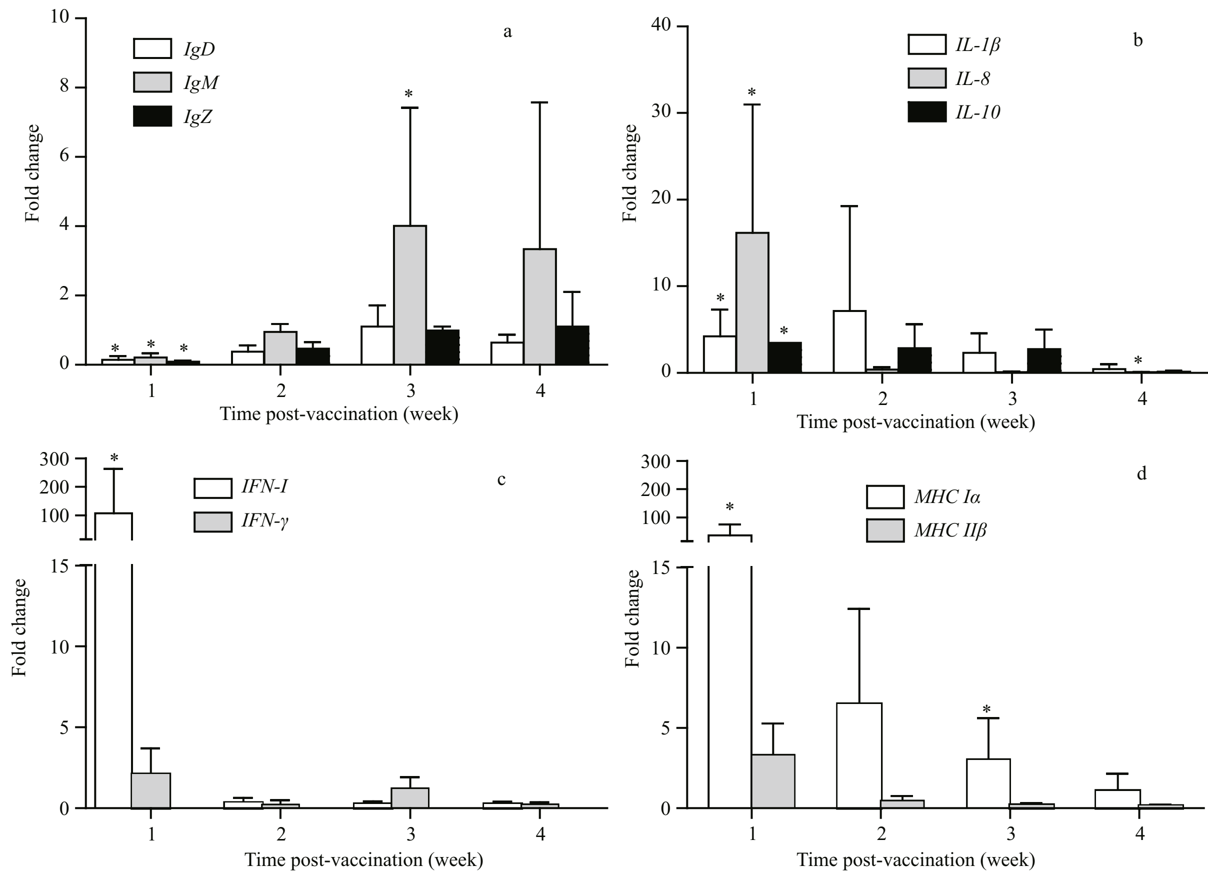

Fig.5 Expression of immune genes, *IgM*, *IgD* and *IgZ* (a), *IL-1β*, *IL-8* and *IL-10* (b), *IFN* and *IFN-γ* (c), and *MHC Iα* and *MHC IIβ* (d) in grass carp, as quantified by real-time quantitative PCR following vaccination of fish with the recombinant fusion protein

The mRNA level of each immune gene was normalized against β-actin. Data represent the mean±S.D. of three assays. Significant differences between the control and vaccination groups are indicated by * at $P<0.05$.

in the fish was observed on the second day post-challenge. The clinical signs were swelling at the injection site, and fin and gill necrosis in the morbid fish, and the pathogenic bacterium *F. columnare* was isolated from the gills and livers.

4 DISCUSSION

Bacterial OMPs are regarded as candidate immunogens for the development of recombinant protein vaccines because their external existence on the bacterial cell surface may facilitate recognition by the host immune system (Lin et al., 2002). It is recognized that recombinant proteins of some OMPs from pathogenic bacteria such as *Vibrio parahaemolyticus* (Mao et al., 2007) and *V. harveyi* (Zhang et al., 2007), *Streptococcus iniae* (Cheng et al., 2010), and *Edwardsiella tarda* (Sun et al., 2011) can induce protective immunity in host species. In a previous study, Sun and Nie (2001) found that some *F. columnare* OMPs were immunogenic, as revealed by Western blotting with fish antiserum. Indeed, the present study has shown that *F. columnare* OMPs are immunogenic and have potential as candidate antigens for the development of vaccines against columnaris disease.

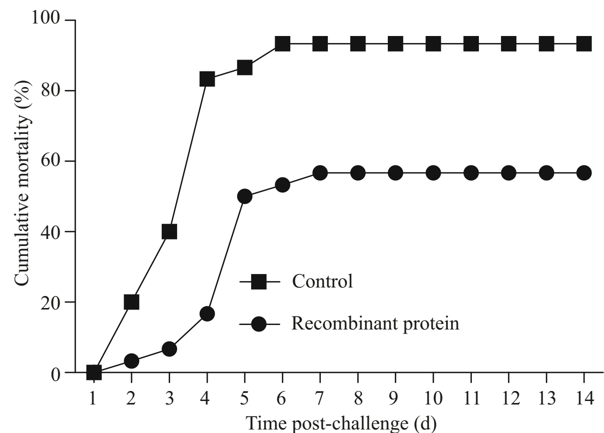

Fig.6 Cumulative mortality after challenge infection with *Flavobacterium columnare* G_4 in grass carp vaccinated 4 weeks previously with the recombinant fusion protein

Fish were vaccinated with recombinant protein (■). Control fish were vaccinated with the pET-32a vector tag-protein (●).

However, virulence factors, despite their pathogenicity, may also serve as promising vaccine candidates. For example, adhesin, which is a type of virulence factor in some pathogens, can be used readily as a target for vaccine development (Wells et al., 2007). Additionally, proteases, together with chondroitin AC lyases and adherence factors, have been reported as virulence factors in *F. columnare* (Newton et al., 1997; Decostere et al., 1999; Xie et al., 2005). In the present study, five possible virulence factors, including chondroitin AC lyase and four proteases (zinc metalloprotease, prolyl oligopeptidase, thermolysin, collagenase), which are *F. columnare* OMPs, were analyzed to determine their antigenic regions, and these antigenic regions, when expressed as a recombinant fusion protein, induced adaptive as well as some innate immune responses in grass carp, thereby providing immune protection against infection with *F. columnare*.

Chondroitin AC lyase can degrade acidic polysaccharides such as hyaluronic acid and chondroitin sulfates, which are components of the extracellular matrix of host tissues (Newton et al., 1997), thus facilitating bacterial invasion (Xie et al., 2005). In *F. columnare*, lyase is highly related with virulence (Suomalainen et al., 2006). It has been shown that the other four OMPs are involved in pathogenicity in other bacterial species. Zinc metalloprotease is distributed widely in eukaryotes and in prokaryotes such as *V. anguillarum* and *V. aestuarianus*; this protein was found to have roles in bacterial invasion and host lethality, and even in influencing host immune responses (Norqvist et al., 1990; Labreuche et al., 2010). Prolyl oligopeptidase, also known as prolyl endopeptidase and post-proline cleaving enzyme, is a member of the serine protease family, and is present in many pathogenic bacteria, such as *A. hydrophila* (Kanatani et al., 1993). Thermolysin, which is very stable in organic solutions and under conditions of high salt or high temperature, may be functional in the survival and reproduction of bacteria under certain conditions (Inouye et al., 1996; Muta and Inouye, 2002). Bacterial collagenases have a broad range of substrates and can degrade natural and denatured collagens (Peterkofsky, 1982). The type II collagenase of *F. psychrophilum*, for example, can degrade gelatin and type II collagen, thus facilitating bacterial invasion and colonization of host species (Ostland et al., 2000). Here, we described the preparation and fusion of the regions encoding the antigenic regions of the five OMPs from *F. columnare*. The expressed recombinant fusion protein had high immunogenicity in grass carp, and when used as a vaccine was able to protect the fish from challenge with *F. columnare*.

Recombinant fusion proteins have been reported to have desirable attributes when used as vaccines. Zhang et al. (2007) found that the recombinant fusion protein, r-OmpK-GAPDH, had a higher level of immunogenicity and provided greater protection against *V. harveyi* than r-OmpK or r-GAPDH alone in the large yellow croaker. The OmpH-like surface antigen can generate a protective immune response in rainbow trout against another bacterial pathogen also in the genus *Flavobacterium*, *F. psychrophilum* (Dumetz et al., 2006). In the present study, the recombinant fusion protein designed on the basis of antigenic regions of OMPs also had a high level of immunogenicity and provided significant protection against *F. columnare* infection in grass carp, although the protective effects of the five OMPs were not examined separately.

The immunogenicity and protective role of the recombinant fusion protein may be a result of the IgM antibody response detected and the expression of immune genes following the intraperitoneal injection of the recombinant protein followed by the challenge infection. In grass carp, as in other teleost fish, three Ig types have been reported (Xiao et al., 2010). In fish, IgM functions in systemic and mucosal immune responses, IgZ/T plays a role in mucosal responses (Zhang et al., 2010; Xu et al., 2013), while the role of IgD is not well-defined (Enholm et al., 2011). Additionally, IgZ in grass carp is very much similar to IgM in terms of its amino acid sequence in this fish (Xiao et al., 2010), and efforts to develop antibodies to recombinant IgZ in grass carp were unsuccessful (own unpublished data); this may have been caused by the high level of similarity between IgM and IgZ in grass carp (Xiao et al., 2010). In the present study, fish serum was examined for IgM only following immunization (Fig.4). A significant increase in IgM antibody levels was observed at 3 and 4 wpv (Fig.4), and the higher level of *IgM* gene expression also at 3 and 4 wpv (Fig.5a) may be indicative of an adaptive immune response to the recombinant fusion protein. As fish that have not been exposed to pathogens are not available in aquaculture research, the early increase in the expression of *IgD*, *IgM* and *IgZ* genes at 1 wpv may be explained as a slightly provoked response in these fish. Nevertheless, it is not unreasonable to conclude that fish can develop an

antibody response to the pathogenic bacterium, *F. columnare*. Increases in the expression of *MHC Iα* and *MHC IIβ* genes, which are involved in antigen processing and presentation, followed by adaptive immune responses (Chen et al., 2010), may provide further evidence to support this conclusion. In teleost fish, it was once considered that endogenous and exogenous antigens were presented by *MHC I* and *MHC II* molecules, respectively (see Dijkstra et al., 2001 for a discussion on this). But, exogenous antigens can be offered by MHC class I molecules when they are packed into or connected to a structure that delivers them into the cytoplasm (Dijkstra et al., 2001). A significant increase in the expression of the *MHC Iα* gene was found also in Japanese flounder vaccinated with recombinant Eta2, an exogenous protein (Sun et al., 2011). However, *MHC I* and *MHC II* antigen presentation in fish has received little attention in research, and it would be interesting to characterize the antigens presented by *MHC I* and *MHC II* molecules in teleost fish.

IL and *IFN* genes have also been observed to respond to stimulation by recombinant fusion proteins. *IL-1β*, a typical pro-inflammatory cytokine, has roles in immune cell proliferation, and in leukocyte respiratory burst and phagocytosis (Secombes et al., 2001; Randelli et al., 2008). *IL-8*, a member of the CXC chemokine subfamily, possesses potent neutrophil chemotactic activity (Oehlers et al., 2010). In mammals, *IL-10* is an immunosuppressive cytokine, and is considered to be an inflammatory cytokine in fish (Inoue et al., 2005). Fish have two *IFN* types (type I and II IFNs), which possibly have a wide range of effects in the immune system (Zou and Secombes, 2011). The increased expression of *IL-1β*, *IL-8* and *IL-10*, and *IFN*, *IFN-γ* genes we observed may imply that fish also have innate immunity against *F. columnare*.

The adaptive immune response has been reported in mandarin fish (*Siniperca chuatsi*) against stimulation with inactivated *F. columnare*, with a similar pattern of increase in the expression of *IgM*, *IgD* and *IgZ* genes as that observed in the present study (Tian et al., 2009). Recently, Makesh et al. (2015) examined IgM antibody levels, and *IgM*, *IgD* and *IgT* gene expression in the systemic and mucosal immune systems of rainbow trout following immunization with attenuated *F. psychrophilum*, and found an elevated level of IgM antibodies, and of *IgM*, *IgD* and *IgT* gene expression. To meet the challenge of developing a fish vaccine against *F. columnare*, further research should be carried out to examine other possible vaccine candidate molecules in *F. columnare* and to understand the global adaptive and innate immune response of fish in the process of vaccination and challenge infections.

References

Al-Harbi A H, Truax R, Thune R L. 2000. Production and characterization of monoclonal antibodies against tilapia *Oreochromis niloticus* immunoglobulin. *Aquaculture*, **188**(3-4): 219-227.

Barnes M E, Bergmann D, Jacobs J, Gabel M. 2009. Effect of *Flavobacterium columnare* inoculation, antibiotic treatments and resident bacteria on rainbow trout *Oncorhynchus mykiss* eyed egg survival and external membrane structure. *J. Fish Biol.*, **74**(3): 576-590.

Chen W H, Jia Z H, Zhang T, Zhang N Z, Lin C Y, Gao F, Wang L, Li X Y, Jiang Y N, Li X, Gao G F, Xia C. 2010. MHC class I presentation and regulation by IFN in bony fish determined by molecular analysis of the class I locus in grass carp. *J. Immunol.*, **185**(4): 2 209-2 221.

Cheng S, Hu Y H, Jiao X D, Sun L. 2010. Identification and immunoprotective analysis of a *Streptococcus iniae* subunit vaccine candidate. *Vaccine*, **28**(14): 2 636-2 641.

Darwish A M, Mitchell A J. 2009. Evaluation of diquat against an acute experimental infection of *Flavobacterium columnare* in channel catfish, *Ictalurus punctatus* (Rafinesque). *J. Fish Dis.*, **32**(5): 401-408.

Declercq A M, Haesebrouck F, van den Broeck W, Bossier P, Decostere A. 2013. Columnaris disease in fish: a review with emphasis on bacterium-host interactions. *Vet. Res.*, **44**(1): 27.

Decostere A, Haesebrouck F, Charlier G, Ducatelle R. 1999. The association of *Flavobacterium columnare* strains of high and low virulence with gill tissue of black mollies (*Poecilia sphenops*). *Vet. Microbiol.*, **67**(4): 287-298.

Decostere A, Haesebrouck F, Devriese L A. 1997. Shieh medium supplemented with tobramycin for selective isolation of *Flavobacterium columnare* (*Flexibacter columnaris*) from diseased fish. *J. Clin. Microbiol.*, **35**(1): 322-324.

Dijkstra J M, Fischer U, Sawamoto Y, Ototake M, Nakanishi T. 2001. Exogenous antigens and the stimulation of MHC class I restricted cell-mediated cytotoxicity: possible strategies for fish vaccines. *Fish Shellfish Immunol.*, **11**(6): 437-458.

Dumetz F, Duchaud E, LaPatra S E, Marrec C L, Claverol S, Urdaci M C, Hénaff M L. 2006. A protective immune response is generated in rainbow trout by an OmpH-like surface antigen (P18) of *Flavobacterium psychrophilum*. *Appl. Environ. Microb.*, **72**(7): 4 845-4 852.

Enholm E S, Bengten E, Wilson M. 2011. Insights into the function of IgD. *Dev. Comp. Immunol.*, **35**(12): 1 309-1 316.

Figueiredo H C P, Klesius P H, Arias C R, Evans J, Shoemaker C A, Pereira D J Jr, Peixoto M T D. 2005. Isolation and

characterization of strains of *Flavobacterium columnare* from Brazil. *J. Fish Dis*., **28**(4): 199-204.

Inoue Y, Kamota S, Ito K, Yoshiura Y, Ototake M, Moritomo T, Nakanishi T. 2005. Molecular cloning and expression analysis of rainbow trout (*Oncorhynchus mykiss*) interleukin-10 cDNAs. *Fish Shellfish Immunol*., **18**(4): 335-344.

Inouye K, Lee S, Tonomura B I. 1996. Effect of amino acid residues at the cleavable site of substrates on the remarkable activation of thermolysin by salts. *Biochem*. *J*., **315**(1): 133-138.

Kanatani A, Yoshimoto T, Kitazono A, Kokubo T, Tsaru D. 1993. Prolyl endopeptidase from *Aeromonas hydrophila*: cloning, sequencing, and expression of the enzyme gene, and characterization of the expressed enzyme. *J. Biochem*., **113**(6): 790-796.

Labreuche Y, Roux F L, Henry J, Zatylny C, Huvet A, Lambert C, Soudant P, Mazel D, Nicolas J L. 2010. *Vibrio aestuarianus* zinc metalloprotease causes lethality in the Pacific oyster *Crassostrea gigas* and impairs the host cellular immune defenses. *Fish Shellfish Immunol*., **29**(5): 753-758.

Leal C A G, Carvalho-Castro G A, Sacchetin P S C, Lopes C O, Moraes A M, Figueiredo H C P. 2010. Oral and parenteral vaccines against *Flavobacterium columnare*: evaluation of humoral immune response by ELISA and in vivo efficiency in Nile tilapia (*Oreochromis niloticus*). *Aquacult*. *Int*., **18**(4): 657-666.

Li N, Zhang J, Zhang L Q, Nie P. 2010. Difference in genes between a high virulence strain G_4 and low virulence strain G_{18} of *Flavobacterium columnare* by using suppression subtractive hybridization. *J. Fish Dis*., **33**(5): 403-412.

Lin J, Huang S X, Zhang Q J. 2002. Outer membrane proteins: key players for bacterial adaptation in host niches. *Microbes Infect*., **4**(3): 325-331.

Liu G Y, Nie P, Zhang J, Li N. 2008. Proteomic analysis of the sarcosine-insoluble outer membrane fraction of *Flavobacterium columnare*. *J. Fish Dis*., **31**(4): 269-276.

Liu Z X, Liu G Y, Li N, Xiao F S, Xie H X, Nie P. 2012. Identification of immunogenic proteins of *Flavobacterium columnare* by two-dimensional electrophoresis immunoblotting with antibacterial sera from grass carp, *Ctenopharyngodon idella* (Valenciennes). *J. Fish Dis*., **35**(4): 255-263.

Lu Q Z, Ni D S, Ge R F. 1975. Studies on the gill diseases of the grass carp (*Ctenopharyngodon idellulus*) I. Isolation of a myxobacterial pathogen. *Acta Hydrobiol*. *Sin*., **5**(3): 315-334. (in Chinese with English abstract)

Makesh M, Sudheesh P S, Cain K D. 2015. Systemic and mucosal immune response of rainbow trout to immunization with an attenuated *Flavobacterium psychrophilum* vaccine strain by different routes. *Fish Shellfish Immunol*., **44**(1): 156-163.

Mao Z J, Yu L, You Z Q, Wei Y W, Liu Y. 2007. Cloning, expression and immunogenicty analysis of five outer membrane proteins of *Vibrio parahaemolyticus* zj2003. *Fish Shellfish Immunol*., **23**(3): 567-575.

Muta Y, Inouye K. 2002. Inhibitory effects of alcohols on thermolysin activity as examined using a fluorescent substrate. *J. Biochem*., **132**(6): 945-951.

Newton J C, Wood T M, Hartley M M. 1997. Isolation and partial characterization of extracellular proteases produced by isolates of *Flavobacterium columnare* derived from channel catfish. *J. Aquat*. *Anim*. *Health*, **9**(2): 75-85.

Norqvist A, Norrman B, Wolf-Watz H. 1990. Identification and characterization of a zinc metalloprotease associated with invasion by the fish pathogen *Vibrio anguillarum*. *Infect*. *Immun*., **58**(11): 3 731-3 736.

Oehlers S H B, Flores M V, Hall C J, O'toole R, Swift S, Crosier K E, Crosier P S. 2010. Expression of zebrafish *cxcl*8 (interleukin-8) and its receptors during development and in response to immune stimulation. *Dev*. *Comp*. *Immunol*., **34**(3): 352-359.

Olivares-Fuster O, Terhune J S, Shoemaker C A, Arias C R. 2010. Cloning, expression, and immunogenicity of *Flavobacterium columnare* heat shock protein DnaJ. *J. Aquat*. *Anim*. *Health*, **22**(2): 78-86.

Ostland V E, Byrne P J, Hoover G, Ferguson H W. 2000. Necrotic myositis of rainbow trout, *Oncorhynchus mykiss* (Walbaum): proteolytic characteristics of a crude extracellular preparation from *Flavobacterium psychrophilum*. *J. Fish Dis*., **23**(5): 329-336.

Peterkofsky B. 1982. Bacterial collagenase. *Methods Enzymol*., **82**: 453-471.

Poobalane S, Thompson K D, Ardó L, Verjan N, Han H J, Jeney G, Hirono I, Aoki T, Adams A. 2010. Production and efficacy of an *Aeromonas hydrophila* recombinant S-layer protein vaccine for fish. *Vaccine*, **28**(20): 3 540-3 547.

Randelli E, Buonocore F, Scapigliati G. 2008. Cell markers and determinants in fish immunology. *Fish Shellfish Immunol*., **25**(4): 326-340.

Secombes C J, Wang T, Hong S, Peddie S, Crampe M, Laing K J, Cunningham C, Zou J. 2001. Cytokines and innate immunity of fish. *Dev*. *Comp*. *Immunol*., **25**(8-9): 713-723.

Shoemaker C A, Klesius P H, Drennan J D, Evans J J. 2011. Efficacy of a modified live *Flavobacterium columnare* vaccine in fish. *Fish Shellfish Immunol*., **30**(1): 304-308.

Smith P K, Krohn R I, Hermanson G T, Mallia A K, Gartner F H, Provenzano M D, Fujimoto E K, Goeke N M, Olson B J, Klenk D C. 1985. Measurement of protein using bicinchoninic acid. *Anal*. *Biochem*., **150**(1): 76-85.

Sun B J, Nie P. 2001. Outer membrane protein and lipopolysaccharide of *Cytophaga columnaris* and their immunogenicity to the mandarin fish, *Siniperca chuatsi*. *Acta Hydrobiol*. *Sin*., **25**(5): 524-527. (in Chinese with English abstract)

Sun Y, Liu C S, Sun L. 2011. Comparative study of the immune effect of an *Edwardsiella tarda* antigen in two forms: subunit vaccine vs DNA vaccine. *Vaccine*, **29**(11): 2 051-2 057.

Suomalainen L R, Tiirola M, Valtonen E T. 2006. Chondroitin AC lyase activity is related to virulence of fish pathogenic *Flavobacterium columnare*. *J. Fish Dis*., **29**(12): 757-763.

Tian J Y, Sun B J, Luo Y P, Zhang Y G, Nie P. 2009. Distribution of IgM, IgD and IgZ in mandarin fish, *Siniperca chuatsi* lymphoid tissues and their transcriptional changes after *Flavobacterium columnare* stimulation. *Aquaculture*, **288**(1-2): 14-21.

Wang L F, Xie H X, Zhang J, Li N, Yao W J, Zhang L Q, Xiong C X, Nie P. 2010. Columnare disease and genetic diversity of its bacterial pathogen *Flavobacterium columnare* in freshwater fish in China. *Acta Hydrobiol. Sinica*, **34**(2): 367-377. (in Chinese with English abstract)

Wang N, Yang Z, Zang M F, Liu Y J, Lu C P. 2013. Identification of Omp38 by immunoproteomic analysis and evaluation as a potential vaccine antigen against *Aeromonas hydrophila* in Chinese breams. *Fish Shellfish Immunol*., **34**(1): 74-81.

Wells T J, Tree J J, Ulett G C, Schembri M A. 2007. Autotransporter proteins: novel targets at the bacterial cell surface. *FEMS Microbiol. Lett*., **274**(2): 163-172.

Xiao F S, Wang Y P, Yan W, Chang M X, Yao W J, Xu Q Q, Wang X X, Gao Q, Nie P. 2010. Ig heavy chain genes and their locus in grass carp *Ctenopharyngodon idella*. *Fish Shellfish Immunol*., **29**(4): 594-599.

Xie H X, Nie P, Chang M X, Liu Y, Yao W J. 2005. Gene cloning and functional analysis of glycosaminoglycan-degrading enzyme chondroitin AC lyase from *Flavobacterium columnare* G_4. *Arch. Microbiol*., **184**(1): 49-55.

Xie H X, Nie P, Sun B J. 2004. Characterization of two membrane-associated protease genes obtained from screening out-membrane protein genes of *Flavobacterium columnare* G_4. *J. Fish Dis*., **27**(12): 719-729.

Xu Z, Parra D, Gómez D, Salinas I, Zhang Y A, Jørgensen L V G, Heinecke R D, Buchmann K, LaPatra S, Sunyer J O. 2013. Teleost skin, an ancient mucosal surface that elicits gut-like immune responses. *Proc. Nat. Acad. Sci. USA*, **110**(32): 13 097-13 102.

Yan W, Xiao F S, Nie P. 2012. Preparation of polyclonal antibody against IgM, IgZ and IgD in grass carp (*Ctenopharyngodon idellus*) and expression analysis in tissues. *Acta Hydrobio. Sin*., **36**(1): 35-40. (in Chinese with English abstract)

Zhang C, Yu L, Qian R. 2007. Characterization of OmpK, GAPDH and their fusion OmpK-GAPDH derived from *Vibrio harveyi* outer membrane proteins: their immunoprotective ability against vibriosis in large yellow croaker (*Pseudosciaena crocea*). *J. Appl. Microbiol*., **103**(5): 1 587-1 599.

Zhang Y A, Salinas I, Li J, Parra D, Bjork S, Xu Z, LaPatra S E, Bartholomew J, Sunyer J O. 2010. IgT, a primitive immunoglobulin class specialized in mucosal immunity. *Nat. Immunol*., **11**(9): 827-835.

Zhang Z H, Wu H Z, Xiao J, F Wang Q Y, Liu Q, Zhang Y X. 2012. Immune responses of zebrafish (*Danio rerio*) induced by bath-vaccination with a live attenuated *Vibrio anguillarum* vaccine candidate. *Fish Shellfish Immunol*., **33**(1): 36-41.

Zou J, Secombes C J. 2011. Teleost fish interferons and their role in immunity. *Dev. Comp. Immunol*., **35**(12): 1 376-1 387.