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Original research article

# Immunogenic proteins and their vaccine development potential evaluation in outer membrane proteins (OMPs) of *Flavobacterium columnare*

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ABSTRACT

*Flavobacterium columnare* is a Gram-negative bacterium that causes columnaris disease in freshwater fish worldwide. Many studies have focused on the identification of protective antigens to aid in the development of novel vaccines against the disease. In this study, an immunoblotting approach was employed to identify immunogenic outer membrane proteins (OMPs) from *F. columnare* in two-dimensional electrophoresis (2-DE) map gels using antibacterial sera obtained from grass carp (*Ctenopharyngodon idella*), and anti-grass carp-recombinant Ig (rIg) monoclonal antibodies. Five unique immunogenic proteins, including the gliding motility lipoprotein GldJ (GldJ), hypothetical protein FCOL_13420 (Fco1), lipoprotein (Lip), F0F1 ATP synthase subunit beta (F0f1) and outer membrane efflux protein precursor (Omep), were characterized. Over-expression of these proteins in *Escherichia coli* DE3, and their immunogenicity and protective efficacy were evaluated in grass carp. The relative percent survival (RPS) of the groups immunized separately with recombinant GldJ, Lip and Omep was 72%, 64% and 68%, respectively when compared to control fish. Up-regulation of immuno-related genes and specific antibodies were detected in immunized fish and sera of immunized fish inhibited the growth of *F. columnare*. The results suggest that GldJ, Lip and Omep are major protective antigens and may be considered as novel candidates in the development of vaccines against columnaris disease in fish.


## 1. Introduction

In freshwater fish, the pathogen *Flavobacterium columnare*, a Gram-negative rod-shaped bacterium, is the causative agent of columnaris disease with severe economic losses in aquaculture worldwide (Declercq, Haesebrouck, den Broeck, Bossier, & Decostere, 2013; Wang et al., 2010). Early since its identification (Davis, 1922) significant efforts have been made to understand its pathogenicity and to develop strategies for prevention and disease treatment (for a review see Declercq et al. (2013)). Recently, a newly developed gene deletion system has been applied successfully to examine the virulence of two chondroitin lyase genes in *F. columnare* (Li et al., 2015). To control disease occurrence in aquaculture systems, preventive measures in management and water treatment and some curative approaches using antimicrobial agents and chemicals have been used. Vaccination has also been efficacious in the prevention of this bacterial disease (Declercq et al., 2013) and immersion of carp (*Cyprinus carpio*) and channel catfish (*Ictalurus punctatus*) in a bacterin resulted in reduced mortality (Liewes, Van Dam, Vos-Maas, & Bootsma, 1982; Moore, Eimers, & Cardella, 1990). However, oral immunization of formalin- or heat-killed bacteria provided higher level of protection (Fujihara & Nakatani, 1971; Ransom, 1975), and intraperitoneal injection of formalin-killed bacteria in Freund's complete adjuvant caused significant systemic humoral response in the Nile tilapia (*Oreochromis niloticus*) (Grabowski, LaPatra, & Cain, 2004). Recently, Shoemaker, Klesius, Drennan, and Evans (2011) developed a modified-live rifampicin-resistant mutant of *F. columnare*, which provided improved protection against columnaris disease in the channel catfish and largemouth bass (*Micropterus salmoides*). Bacterial outer membrane proteins (OMPs) as vaccine candidates have

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received much attention, because their exposed epitopes on cell surface allow them to be recognized easily by the host immune system (Li et al., 2008; Wang et al., 2012). Components of OMPs in *F. columnare* were capable of evoking an immune response and providing immune protection against *F. columnare* infection in Bluntnose black bream (*Megalobrama amblycephala*) and in channel catfish (Hu, Wang, & Chen, 2010; Xia, Wang, & Chen, 2007). In addition, Sun and Nie (2001) have detected four immunogenic protein bands from OMPs of *F. columnare* by SDS-PAGE and Western blotting using antisera obtained from mandarin fish (*Siniperca chuatsi*), but they have not further identified the exact proteins in the bands. Using host antisera several immunogenic OMPs in *F. columnare* (Xie, 2005; Liu, Nie, Zhang, & Li, 2008, Liu, Liu, Li, Xiao, Xie, Nie, 2012) have been identified and recently, Luo et al. (2016), using recombinant fusion protein of five OMPs, including zinc metalloprotease, prolyl oligopeptidase, thermolysin, collagenase and chondroitin AC lyase from *F. columnare*, observed a good level of protection against infection and an increase in the expression of immunoglobulin (Ig) and cytokine genes.

In the present study, using anti-sera collected from *F. columnare* infected fish five OMPs immunogenic proteins of *F. columnare* were identified using two-dimensional electrophoresis (2-DE) and Western blotting analyses. Their role in protection of fish against bacterial infection and in eliciting an immune response was examined.

## 2. Materials and methods

### 2.1. Bacteria and culture conditions

*F. columnare* $G_4$ strain isolated originally from the grass carp with gill-rot disease (Lu, Ni, & Ge, 1975) routinely used in research (Li et al., 2015; Liu et al., 2012, 2008) was employed in this study. The bacterium was cultivated at 28 °C with gentle agitation in Shieh broth medium as described by Decostere, Haesebrouck, and Devriese (1997). *Escherichia coli* TOP10 and DE3 strains were cultured at 37 °C in Luria-Bertani (LB) broth media.

### 2.2. Preparation of fish antisera against F. columnare and anti-IgM, IgD and IgZ antibodies

In order to ensure infection and to obtain sufficient antisera, thirty grass carp weighing approximately 200 g each were infected twice with an intraperitoneal injection of 100 μL *F. columnare* $G_4$ suspension ($10^8$ colony forming unit (CFU)/mL) with one week interval. Four weeks after the last injection, all fish were anaesthetized with tricaine methanesulphonate (MS 222) and blood was collected from the caudal vein and subsequently stored overnight at −4 °C. The antisera were mixed and stored at −70 °C before being used.

The recombinant proteins of grass carp IgM, IgD and IgZ were expressed and purified as reported by Liu et al. (2012). The hybridoma secreting monoclonal antibodies (mAbs) against grass carp IgM, IgD and IgZ were established by fusing SP2/0 with spleen cells from BALB/c mice immunized with the purified IgM, IgD and IgZ recombinant proteins, respectively. Mouse-anti-grass carp IgM, IgD and IgZ mAbs were obtained according to Heijden, Rooijakkers, Booms, Rombout, and Boon (1995) and Al-Harbi, Truax, and Thune (2000), and verification of mAbs against IgM was reported in a previous study (Luo et al., 2016). mAbs against IgD and IgZ were also verified (data not shown).

### 2.3. Preparation of outer membrane proteins from F. columnare $G_4$

OMPs of *F. colunmare* $G_4$ were prepared according to the method described by Liu et al. (2008). Briefly, a 1000 mL culture of *F. colunmare* (as described above) was harvested by centrifugation at 6000 g for 30 min and washed three times with phosphate buffered saline (PBS; 0.15 M, pH 7.5). The bacterial pellet were resuspended in 20 mL Tris—HCl (20 mM, pH 7.5) and then sonicated at 10 W for 30 min with 30 s intervals. Cellular debris were removed by centrifugation at 4000 g for 15 min. The supernatant was further centrifuged at 100 000 *g* for 1 h at 4 °C. The pellet was resuspended in 20 mL Tris-HCl of 2% (w/v) sodium lauryl sarcosinate and incubated at 25 °C for 30 min to solubilize the inner membrane. The solution was centrifuged at 100 000 *g* for 1 h at 4 °C and the pellet was dissolved in sterile water. Protein concentration was determined using the Bradford assay (Bradford, 1976). The OMPs were stored at −80 °C until use.

### 2.4. Two-dimensional electrophoresis (DE) and immunoblotting

2-D gel electrophoresis was performed according to the procedure described by Liu, Cheng, and Nie (2007) and Wang, Yang, Zang, Liu, and Lu (2013). Briefly, 200 μg of OMPs were dissolved in 125 μL rehydration buffer (5 M urea, 2 M Thiourea, 2% CHAPS, 1% DTT, 2% SB3-10, 0.2% Biolyte Ampholyte, 20 mM Tris, 0.0002% Bromophenol Blue) and then rehydrated in a pH 3—10 nonlinear immobilized pH gradient (IPG) strip (7 cm length, Bio-Rad) at 22 °C for 12 h. Isoelectric focusing (IEF) was performed in IPG strip as the following: S1: 250 V, 30 min; S2: 1000 V, 1 h; S3: 4000 V, 3 h; and S4: 4000 V, 25,000 Vh on a Bio-Rad Protean IEF Cell (Bio-Rad). Strips were equilibrated in equilibration buffer I (6 M urea; 50 mM Tris; 2% SDS; 20% Glycerol; 2% Dithiothreitol) and then in buffer II (6 M urea; 50 mM Tris; 2% SDS; 20% glycerol; 2.5% iodoacetamide) for 15 min each. Separation in the 2D was performed on SDS-PAGE (12% polyacrylamide) under a constant voltage of 120 V until Bromophenol Blue reached the bottom of gel. Protein spots were visualized with Coomassie brilliant blue G-250 staining and gels were scanned using an image scanner.

Western blotting was performed as described in Liu et al. (2012). OMPs separated by 2-DE were electroblotted on a nitrocellulose filter (Millipore, USA). Blocked for 1 h at room temperature with 1× PBS containing 5% non-fat dry milk, the nitrocellulose filter was incubated with primary antibody (grass carp anti-*F. columnare* $G_4$ sera, 1:100 dilution) and secondary antibodies (mouse anti-recombinant IgM, IgD and IgZ mAbs, respectively, 1:5000 dilution). Fish sera collected from non-infected fish were used as control. Between incubations, membranes were washed three times (5 min each) in 1× PBS containing 0.05% Tween 20 (PBST), before being incubated with goat-anti-mouse IgG conjugated with horseradish peroxidase (HRP) (1:5000 dilution). The antibody-bound proteins were detected using SuperSignal West Pico Trial kit (Thermo, USA) and ECL Western blot system (Bio-Rad, USA) according to the manufacturers' instructions.

### 2.5. Identification of immunogenic proteins

The OMPs recognized by grass carp anti-*F. columnare* sera were excised from the 2-D gels. The proteins were destained, dehydrated, rehydrated and incubated as described by Liu et al. (2012). Proteins were identified using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/TOF MS). Peptide mass fingerprints coupled with peptide fragmentation patterns were used to identify homologue proteins at the National Center for Biotechnology Information (NCBI) nonredundant (nr)

database (http://www.ncbi.nlm.nih.gov/) using the Mascot search engine (Li et al., 2012; Ni, Wang, Liu, & Lu, 2010).

### 2.6. Cloning and recombinant protein production

The genes encoding immunogenic proteins were retrieved from GenBank database, and the signal peptide sequences were predicted using the SignalP 3.0 server. Based on the results, five pairs of primers were designed to amplify the coding sequences including appropriate enzymatic restriction sites at the 5′ ends to allow the correct expression of each proteins (Table 1). The five PCR products were cloned into plasmid pET-28a, confirmed by sequencing and expressed in *E. coli* DE3 strain. For proteins expression, *E. coli* DE3 harbouring recombinant plasmids were incubated at 37 °C until reached 0.6 optical density ($OD_{600}$). The recombinant protein expression was induced with 0.2 mM IPTG under the optimization of expression conditions. After being harvested, bacterial cells were washed three times with PBS, and then resuspended in PBS for sonication in ice bath, and inclusion bodies were solubilized in denaturing solution, before being purified using the Ni-NTA based affinity chromatography. Concentration of purified proteins were determined using the Bradford method, and protein samples stored at −20 °C until use.

### 2.7. Fish immunization and challenge infection

Grass carp, weighing 40–60 g each, were purchased from a commercial fish farm in Hubei Province, China, and were acclimatized at 25 °C for at least three weeks before experiment. One hundred and eighty fish were divided randomly into six groups, each with thirty individuals. Individuals in groups one to five were intraperitoneally injected with 100 μg of each recombinant proteins, i.e. gliding motility lipoprotein GldJ (rGldJ), hypothetical protein FCOL_13420 (rFcol), lipoprotein (rLip), F0F1 ATP synthase subunit beta (rF0f1) and outer membrane efflux protein precursor (rOmep), respectively. Fish in group six were the control group and were injected with 0.2 mL 0.01 M PBS (pH 7.4). Fish were maintained in 50 L tanks and fed with commercial food pellets (2% body weight daily). 28 days post-immunization (dpi), fish were challenged with the intraperitoneal injection of 0.2 ml *F. columnare* $G_4$ suspension ($5 \times 10^8$ CFU/mL) and observed daily during 15 days. Dead fish were necropsied, and *F. columnare* infection was confirmed by PCR, as reported by Bader, Nusbaum, and Shoemaker (2003). The relative percent survival (RPS) was calculated according to Mao, Yu, You, Wei, and Liu (2007): RPS = 1 − (mortality in vaccinated fish/mortality in control fish) × 100%.

### 2.8. Determination of antibody responses in immunized fish

Fish were anaesthetized and blood samples were collected at 7, 14, 21, 28 dpi from caudal vein of three fish in each group using a syringe. Blood was allowed to clot overnight at 4 °C to collect sera. Antibody level in sera was determined by ELISA as described by Ni et al. (2010). Briefly, 96-well plates were coated with the purified recombinant proteins (5–10 μg/mL). Subsequently, 2-fold serial dilutions of the sera with the first dilution of 1:8 were added to the 96-well plates. Antibodies binding the antigen were detected using mouse anti-recombinant grass carp IgM, IgD and IgZ mAbs (1:5000 diluted in PBS) respectively, followed by goat-anti-mouse IgG conjugated with HRP (1:5000 diluted in PBS). The colour was developed with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate for 30 min and the reaction stopped with 2.0 M $H_2SO_4$. The plates were read using a microplate reader at 450 nm.

### 2.9. Western blotting

The recombinant proteins, rGldJ, rFcol, rF0f1, rlip and rOmep were electroblotted on a 0.45 μm PVDF membrane. The membrane was blocked in 1× PBS with 5% skimmed milk and incubated with sera (1:100 diluted in PBS) raised from infected grass carp. Anti-grass carp IgM, IgD and IgZ mAbs (1:5000 diluted in PBST) were used respectively as secondary antibodies, and goat-anti-mouse IgG conjugated with HRP was (1:5000 diluted in PBST) used as

**Table 1**
Primers list.

| Gene name | Primer | Sequence (5′ → 3′) |
|---|---|---|
| For construction of recombinant plasmid | | |
| gliding motility lipoprotein GldJ | GldJ-f | CCGGAATTCAAGTCTAGTACTAAAGACAC (*Eco*R I) |
| | GldJ-r | CCCAAGCTTTTATTTTCTATTTCTTGGTG (*Hind* III) |
| hypothetical protein FCOL_13420 | fcol-f | CCGGAATTCGATTATAATACGCTAGGCGC (*Eco*R I) |
| | fcol-r | CCGCTCGAGTTAATTTCGTTTAGTATAGC (*Xho* I) |
| lipoprotein | lip-f | CGCGGATCCCATTGCAAAAAAAACGAAAC (*Bam*H I) |
| | lip-r | CCCAAGCTTTTTTTTAGTATAATCTCCCGC (*Hind* III) |
| F0F1 ATP synthase subunit beta | f0f1-f | CGCGGATCCATGTCTAAGGTAATAGGAAAAGTTGC (*Bam*H I) |
| | f0f1-r | CCCAAGCTTTGCTTCAGCTAACATTTTTTGTCC (*Hind* III) |
| outer membrane efflux protein precursor | omepp-f | CCGGAATTCAAAATGAACTATTCATTTAG (*Eco*R I) |
| | omepp-r | CCGCTCGAGGTTCTTAAATAATAGTTTTTC (*Xho* I) |
| For Real-time PCR detection | | |
| β-actin | β-actin-f | AGAGTATTTACGCTCAGGTGGG |
| | β-actin-r | CCTTCTTGGGTATGGAGTCTTG |
| IL-1β | IL-1β-f | GGAGAATGTGATCGAAGAGCGT |
| | IL-1β-r | GCTGATAAACCATCCGGGA |
| IL-10 | IL-10-f | ACTGACTGTTGCTCATTTGTGG |
| | IL-10-r | TACTGCTCGATGTACTTAAAGAG |
| CRP | CRP-f | GTGTGGATGACAATGTGTGTGAAG |
| | CRP-r | AGGAACCGTATGTGCCAAAGGGAA |
| MHC IIB | MHC IIB-f | TACTACCAGATTCACTCGG |
| | MHC IIB-r | CGGGTTCCAGTCAAAGAT |
| IFN-I | IFN-I-f | GGTGAAGTTTCTTGCCCTGACCTTAG |
| | IFN-I-r | CCTTATGTGATGGCTGGTATCGGG |
| IFN-γ | IFN-γ-f | AGAAACCCTATGGGCGATTA |
| | IFN-γ-r | CTGCCTCTTGATGCTTTTGG |

Note: Underlined nucleotides are restriction sites for the enzymes indicated in the brackets; f-forward primer; r-reverse primer.

bound antibody. The immunoreactive bands were detected as described above.

### 2.10. Quantitative real-time PCR

Blood samples were obtained from three fish from each group at 1 and 7 dpi. Total RNA (tRNA) samples were isolated using Trizol Reagent (Invitrogen) and treated with DNase I (Fermentas) to eliminate potential genomic DNA contaminant. The cDNA was then synthesized from 0.5 μg tRNA using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's protocol. Quantitative real time PCR (qRT-PCR) was run on the Bio-Rad CFX96™ Real-Time System (Bio-Rad). Amplifications were carried out using primers listed in Table 1 for a final volume of 20 μL, containing 1 μL of each primers (0.5 μM), 6 μL $ddH_2O$, 2 μL template, and 10 μL 2 × iQTM SYBR Green Supermix (Bio-Rad). All samples were tested in triplicate according to the following cycle: 3 min at 95 °C, followed by 45 cycles consisting of 20 s at 95 °C, 20 s at 55 °C and 30 s at 72 °C. Relative gene expression level was determined by comparative threshold cycle method ($2^{-\Delta\Delta C_t}$) with β-actin as reference gene, as used by Zhang et al. (2012).

### 2.11. Serum-mediated antibacterial activity

This activity test was performed based on the method reported by Caipang, Hynes, Puangkaew, Brinchmann, and Kiron (2008). Briefly, blood was collected from the caudal vein of grass carp at four weeks post-immunization (wpi), and serum was collected as described above. An overnight culture of *F. columnare* $G_4$ was adjusted to $10^6$ CFU/mL in 1× PBS. 50 mL bacterial suspension and an equal volume of the sera were transferred to a 0.5 mL sterile tube. The solution was mixed for 3 h incubation at 28 °C and was serially diluted and plated in triplicate on Shieh agar plates to count the quantity of *F. columnare*.

### 2.12. Statistical analysis

Data was expressed as the mean ± S.D. Significant difference in antibody titre, transcript level of immune genes, and serum-mediated antibacterial activity were determined using the Student's *t*-test ($P < 0.05$).

## 3. Results

### 3.1. Immunogenic proteins recognized by the grass carp *F. columnare* antisera

From the 2-D gel, 196 protein spots were detected, most of which ranged from 10 to 130 kDa in molecular weight and 5.0–8.0 in *p*I values (Fig. 1A). Seven immunogenic protein spots were specifically recognized by anti-rIgM mAb, two of which by anti-rIgD mAb and two by anti-rIgZ mAb (Fig. 1B–D). No immune reactive spots were detected when mixed antisera obtained from non-immunized grass carp was used as control (data not shown). These proteins were identified as gliding motility lipoprotein J (GldJ), hypothetical protein FCOL_13420 (Fcol), F0F1 ATP synthase

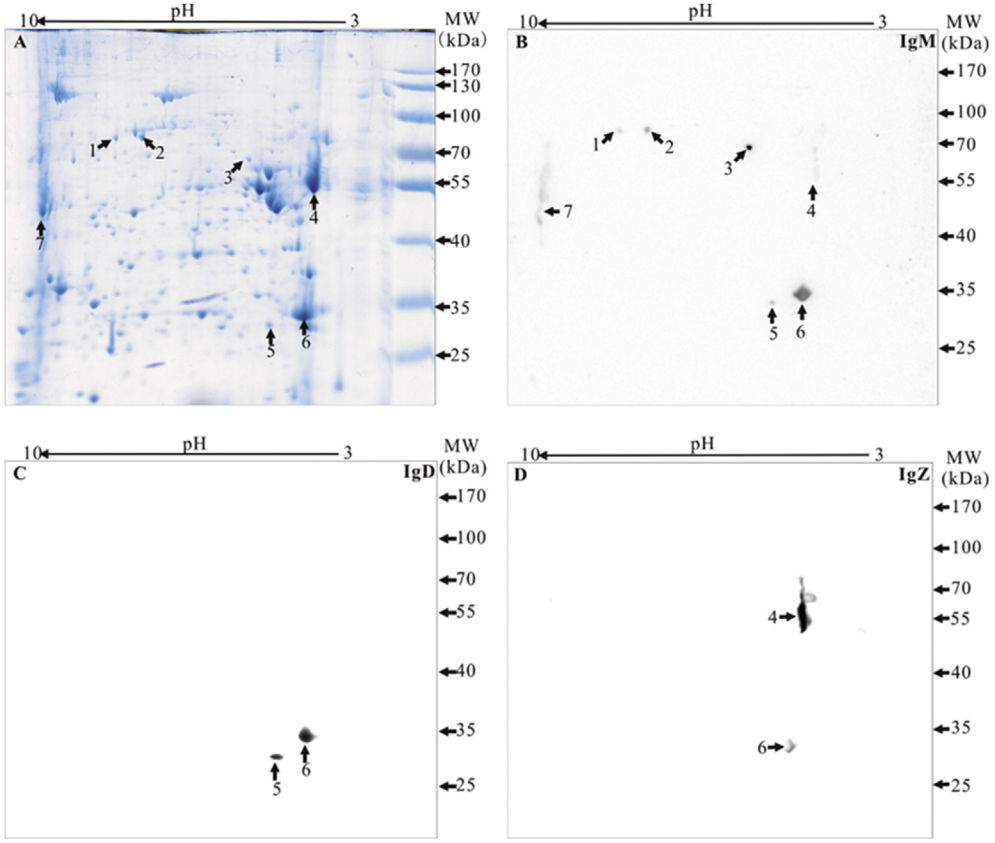


**Fig. 1.** Out member proteins (OMPs) of *Flavobacterium columnare* $G_4$ strain separated on 2-dimensional electrophoresis (2-DE) gels and recognized by grass carp antibacterial sera and anti-grass carp-Ig monoclonal antibodies (mAbs). (A) 2-DE profile of OMPs of *F. columnare* $G_4$, showing the seven proteins recognized by anti-grass carp sera; (B) Immunogenic proteins recognized by anti-grass carp-recombinant IgM (rIgM) mAb; (C) Immunogenic proteins recognized by anti-grass carp-recombinant IgD (rIgD) mAb; (D) Immunogenic proteins recognized by anti-grass carp-recombinant IgZ (rIgZ) mAb. Mw: molecular mass; *p*I, isoelectric point.

**Table 2**
Immunogenic proteins in out member proteins (OMPs) of *Flavobacterium columnare* identified by MALDI-TOF/TOF MS.

| Spot number | Protein name | NCBI accession no./species | Theoretical *p*I/Mw (kDa) | Mascot score | Sequence coverage | Ig class recognition |
|---|---|---|---|---|---|---|
| 1 | gliding motility lipoprotein GldJ | gi\|365961208/*F. columnare* | 9.09/65137 | 781 | 24% | IgM |
| 2 | gliding motility lipoprotein GldJ | gi\|365961208/*F. columnare* | 9.09/65137 | 1211 | 35% | IgM |
| 3 | hypothetical protein FCOL_13420 | gi\|365961705/*F. columnare* | 8.16/60953 | 904 | 38% | IgM |
| 4 | F0F1 ATP synthase subunit beta | gi\|365961594/*F. columnare* | 4.86/54162 | 903 | 25% | IgM/IgZ |
| 5 | lipoprotein | gi\|365960181/*F. columnare* | 6.34/28404 | 451 | 27% | IgM/IgD |
| 6 | lipoprotein | gi\|365960181/*F. columnare* | 6.34/28404 | 839 | 41% | IgM/IgD/IgZ |
| 7 | outer membrane efflux protein precursor | gi\|365960961/*F. columnare* | 8.98/49933 | 542 | 19% | IgM |

*p*I, isoelectric point; Mw, molecular mass.

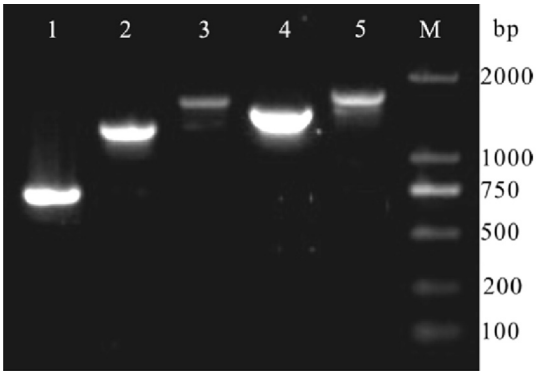


**Fig. 2.** Amplification of five immunogenic protein genes in *Flavobacterium columnare* $G_4$ strain. M: molecular marker; lane 1, lipoprotein, 726 bp; lane 2, outer membrane efflux protein precursor, 1275 bp; lane 3, hypothetical protein FCOL_13420, 1584 bp; lane 4, F0F1 ATP synthase subunit beta, 1527 bp; lane 5, gliding motility lipoprotein GldJ, 1659 bp.

subunit beta (F0f1), lipoprotein (Lip) and outer membrane efflux protein precursor (Omep) (Table 2).

### 3.2. Expression and purification of the recombinant proteins

Five immunogenic proteins *gldJ*, *fcol*, *f0f1*, *lip* and *omep*, without signal coding nucleotides were amplified successfully from *F. columnare* $G_4$ (Fig. 2) and expressed in *E. coil* DE3. All the recombinant proteins were over-expressed at their predicted molecular weights (Fig. 3A), and they were produced as insoluble fusion bodies (data not shown). After His-tag affinity chromatography rFcol, rLip and rF0f1 were purified to single bands confirmed by SDS-PAGE (Fig. 3B). One unexpected additional band with a similar molecular weight to rOmep was also induced and was co-purified (Fig. 3B).

### 3.3. Protection of recombinant proteins in grass carp following infection with F. columnare

Immunized grass carp were challenged with *F. columnare* $G_4$ at 4 wpi, and the results showed that cumulative mortalities of the immunized fish with rGldJ, rFcol, rF0f1, rLip, rOmep and PBS were 23.3%, 76.7%, 56.7%, 30%, 26.7% and 83.3%, respectively. Hence, the RPS of rGldJ, rFcol, rF0f1, rLip and rOmep were 72%, 8%, 32%, 64% and 68%, respectively (Table 3). *F. colunmare* were isolated from the liver, spleen, and kidney of dying animals confirming the infection.

### 3.4. Immune response following vaccination with recombinant proteins

Compared with the control group, a significantly higher level of specific IgM antibody was detected in all immunized groups at 3 to 4 wpi ($P < 0.05$) with the exception of rF0f1-immunized group at 3 wpi (Fig. 4A). Meanwhile, sera from rLip-vaccinated fish had higher specific IgD antibody level at 3 and 4 wpi ($P < 0.05$) (Fig. 4B), and sera from rLip- and rF0f1-vaccinated fish had higher specific IgZ antibody level at 1 wpi ($P < 0.05$) (Fig. 4C).

The immunogenicity of recombinant proteins was further analyzed by Western blotting. Except for rF0f1, the other four recombinant proteins were recognized by sera from immunized fish with the recombinant proteins and then by mouse anti-grass carp IgM mAb (Fig. 5A). Recombinant lip protein was recognized also by the grass carp serum and then by mouse anti-grass carp IgD monoclonal antibody (Fig. 5B). The sera collected from fish immunized with PBS did not react to any of the recombinant proteins (data not shown).

To investigate the effect of recombinant protein immunization on the expression of immune-related genes, qRT-PCR was performed to analyze the transcription level of the genes encoding interleukin 1 (*IL-1β*), *IL-10*, major histocompatibility complex class II B (*MHC IIB*), C-reactive protein (*CRP*), type I interferon (*IFN-I*) and

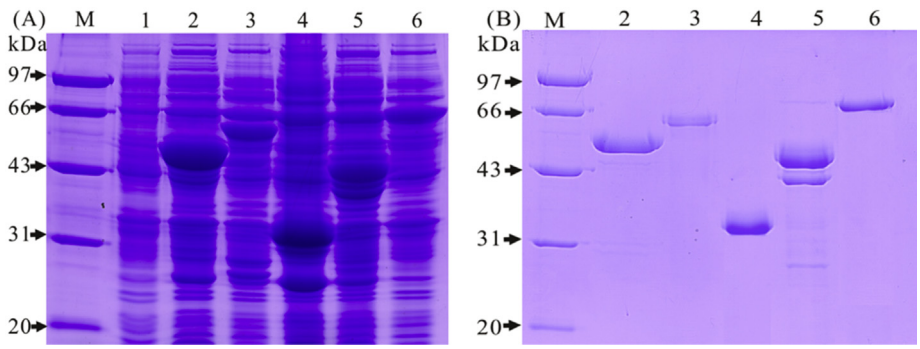


**Fig. 3.** Expression and purification of the five immunogenic proteins in *E. coil* DE3. (A) Protein expression was induced with 0.2 mM IPTG. (B) Purified recombinant proteins after His-tag affinity chromatography. M: molecular marker; lane 1, control (*E. coil* DE3 without recombinant plasmid); lane 2, F0F1 ATP synthase subunit beta, 58.1 kDa; lane 3, hypothetical protein FCOL_13420, 62.2 kDa; lane 4, lipoprotein, 34.5 kDa; lane 5, outer membrane efflux protein precursor, 53.6 kDa; lane 6, gliding motility lipoprotein GldJ, 68.2 kDa.

**Table 3**
Cumulative mortality (CM, %) and relative percent of survival (RPS) of immunized grass carp after infection with *Flavobacterium columnare* $G_4$ strain.

| Immunogen | Dead/Total | CM (%) | RPS (%) |
|---|---|---|---|
| rGldJ | 7/30 | 23.3 | 72 |
| rFcol | 23/30 | 76.7 | 8 |
| rF0f1 | 18/30 | 56.7 | 32 |
| rLip | 9/30 | 30 | 64 |
| rOmep | 8/30 | 26.7 | 68 |
| Control (PBS) | 25/30 | 83.3 | – |

type II interferon (*IFN-γ*) in the blood of immunized fish. The results showed that expression of the majority of the candidate genes was significantly ($P < 0.05$) induced in recombinant protein-vaccinated fish when compared to those in PBS-vaccinated fish at 1 and 7 dpi (Fig. 6).

To further examine the antibacterial activity of sera from infected fish, *F. columnare* were counted on Shieh plates after co-incubation with sera obtained from immunized fish (Fig. 7). The mean bacterial count in PBS group was 320 CFU/mL, and it was not significantly different from the mean bacterial count in the rFco1- or rF0f1-immunized group. However, significant reduction in bacterial count was observed in rOmep-, rLip- and rGldJ-immunized groups ($P < 0.05$).

## 4. Discussion

To identify immunogenic OMPs from *F. columnare*, 2-D electrophoresis and immunoblotting approaches were employed and seven immunogenic protein spots were detected using grass carp anti-*F. columnare* sera and anti-recombinant grass carp Igs mAbs. Seven spots were identified and MALDI-TOF/TOF revealed the identity of five proteins as GldJ, Lip, F0f1, Omep and a hypothetical protein, Fcol which have protective roles against *F. columnare* infection in immunizing grass carp.

In a previous study GldJ, which is located in the outer membrane of *F. columnare*, has been identified also as an immunogenic protein (Xie, 2005). In *F. johnsoniae* it has been shown that GldJ is required for the bacterial gliding motility and for its efficient chitin utilization (Braun & McBride, 2005). Another lipoprotein, Lip was also found as the immunogenic protein in the present study. In fact, lipoproteins in outer membranes of Gram-negative bacteria were considered as potential target antigens for vaccine development as they can serve as pathogen-associated molecular patterns (PAMPs) to initiate immune response via Toll-like receptors (Shivachandra, Kumar, Yogisharadhya, & Viswas, 2014; Thakran et al., 2008; Parra et al., 2010). In *Francisella tularensis*, which is classified as Class A bioterrorism agent causing tularemia, the lipoprotein VacJ

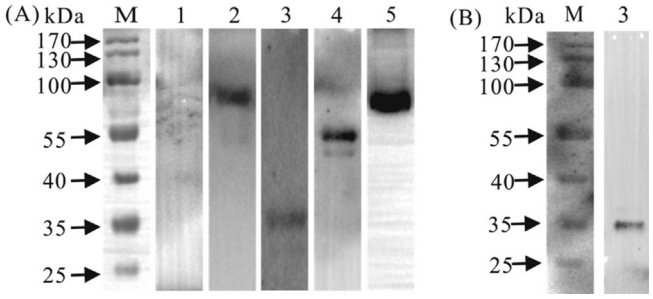


**Fig. 5.** Western blott analysis of the recombinant proteins with sera collected from immunized grass carp 4 weeks post-immunization. M, molecular marker; 1, rF0f1; 2, rFcol; 3, rLip; 4, rOmep; 5, rGldJ. (A) Mouse anti-grass carp rIgM mAb was used as secondary antibody; (B) Mouse anti-grass carp rIgD mAb was used as secondary antibody.

was identified as candidate antigen for vaccine development (Shivachandra et al., 2014). In *Histophilus somni*, an important pathogen of cattle and other ruminants, which causes bovine respiratory disease, the lipoproteins Plp4 and LppB were also reported to induce immune protections (Guzmán-Brambila, Rojas-Mayorquín, Flores-Samaniego, & Ortuño-Sahagún, 2012). On the other hand, outer membrane efflux proteins, which may be involved in antibacterial drug expulsion in Gram-negative bacteria (Poole, 2000), can be also immunogenic and thus considered as protective antigen candidates. Mao et al. (2007) reported that the outer membrane efflux protein, TolC, was a protective antigen and immunogenic protein in *Vibrio parahaemolyticus*. In the present study, an outer membrane efflux protein precursor was identified as an immunogenic protein for *F. columnare*. Meanwhile, two other proteins, F0F1 ATP synthase subunit beta and hypothetical protein FCOL_13420, which were also found to be immunogenic in the present study may require further studies on their bacterial as well as immunogenic functions.

It is reported that immunogenic proteins may stimulate various degrees of neutralization response to microorganisms, and that proteins which can produce higher neutralization ability, are good targets for vaccine development (Chen, Peng, Wang, & Peng, 2004; Cloeckaert, Vizcaıno, Paquet, Bowden, & Elzer, 2002). In order to further investigate the immunogenicity and immune protective effect, the five recombinant proteins were purified to immunize grass carp separately, and significant increase in the expression of pro-inflammatory cytokine genes, including *IL-1β*, *IL-10*, *TNF-a*, *CRP*, and also in *IFN* genes was observed in the blood of grass carp. It is revealed that innate immunity was activated in grass carp following the immunization of each of the five recombinant proteins. An increase in MHC II gene expression was also detected,

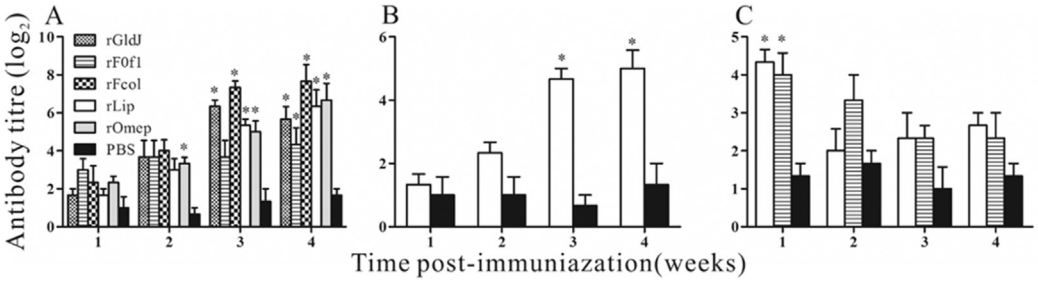


**Fig. 4.** Serum antibody titres of immunized fish. Antigens used for immunization were rGldJ, rF0f1, rFcol, rLip and rOmep. Sera were collected from fish at one to four weeks post-immunization, and mouse anti-grass carp rIgM, rIgD and rIgZ mAbs were used respectively as secondary antibodies. Each column represents the mean of log 2 antibody titres with standard deviation (S.D.) bar. (A) Mouse anti-grass carp rIgM mAb was used as secondary antibody. (B) Mouse anti-grass carp rIgD mAb was used as secondary antibody. (C) Mouse anti-grass carp rIgZ mAb was used as secondary antibody. * indicating significant difference at $P < 0.05$.

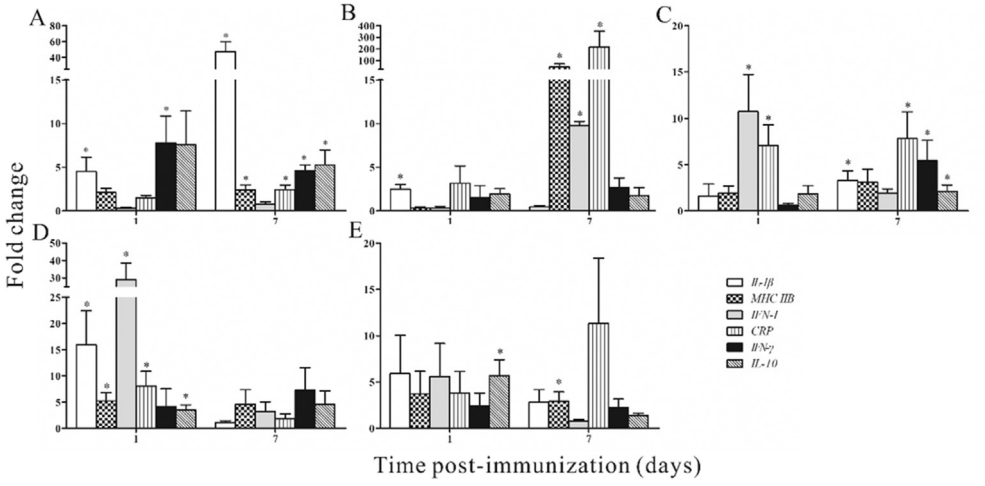


**Fig. 6.** Fold changes expression of immune related genes in the blood of grass carp immunized with recombinant proteins relative to the control. (A to E) represent the five recombinant proteins rGldJ, rFcol, rF0f1, rLip and rOmep, respectively. The asterisks (*) indicate significant differences ($P < 0.05$) among different groups at the same time point; data were expressed as the mean ± S.D, of three individuals.

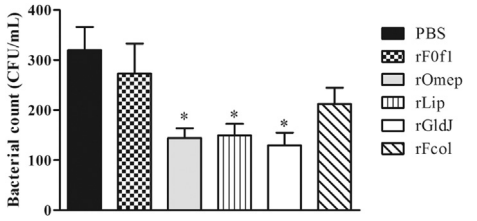


**Fig. 7.** Reduction in *Flavobacterium columnare* counts upon co-incubation with sera collected from vaccinated and PBS-injected (control) grass carps at 4 weeks post-immunization. Bars with asterisks indicate significant differences ($P < 0.05$). Data were expressed as the mean ± S.D. of three individuals.

implying that antigen presenting process and adaptive immune responses may also be activated, and this was further supported by the detected antibody response in the present study. The observed IgM antibody response following the separate immunization of recombinant GldJ, Fcol, F0f1, Lip and Omep proteins may indicate that all the five proteins were antigenic in provoking specific antibody and also innate immune responses. On the other hand, the observed IgD antibody response induced in rLip-immunized grass carp, and IgZ antibody response in rLip-/rF0f1-immunized grass carp may suggest that Lip and/or F0f1 have also a role in inducing more than one type of Igs responses in teleost. However, as revealed in the Western blotting analysis, the fact that the IgM in serum of grass carp immunized with rF0f1 protein failed to recognize the protein, and that the specific IgZ cannot recognize rLip and rF0f1proteins, may imply that the immune response was not strong or specific enough for the antibody-antigen reaction.

Grass carp immunized with rGldJ, rFcol, rF0f1, rLip and rOmep received 72%, 8%, 32%, 64% and 68% RPS, respectively. Although higher antibody levels were induced in rFcol- and rF0f1-immunized groups, a lower protection level was observed for the two groups when challenge-infected with *F. columnare*. This suggests that rFcol or rF0f1 proteins may not be the major antigens to induce neutralization antibody. Similarly, no protection was observed against *F. columnare* when recombinant DnaJ protein was used as antigens although high antibody titers were induced (Olivares-Fuster, Terhune, Shoemaker, & Arias, 2010). According to the result of serum bactericidal activity assay, the serum of grass carp administrated with rGldJ, rLip, rOmep efficiently inhibited the growth of *F. columnare*, implying that GldJ, Lip and Omep were efficacious protective molecules for inducing host immune response against *F. columnare*.

In a previous study, it was reported that three types of Igs, including IgM, IgD and IgZ are present in grass carp (Xiao et al., 2010). In order to identify thoroughly immunogenic proteins in OMPs of *F. colunmnare*, mouse anti-recombinant grass carp IgM, IgD and IgZ mAbs were used. The present study revealed that IgD and IgZ recognized two protein spots in 2-DE and also in ELISA, indicating that IgD and IgZ were involved in the immune response of grass carp against *F. columnare*. The increase in specific IgZ antibody level occurred immediately after the immunization with rF0f1 or rLip, suggesting that IgZ may play an important role in early stage infection in grass carp. The role of IgZ and IgD in systemic immune response against *F. columnare* infection requires certainly further investigations.

It has been reported that OMPs of bacterial pathogens stimulate antibody response in fish. A significant higher IgM antibody titre was detected at 28 (dpi) in fish injected with OMPs of *Edwardsiella tarda*, and OMP assembly factors YaeT and GroEL were found as immunogens (Kumar, Rathore, & Ei-Matbouli, 2014), which confirms the higher Ig titres observed in the present study. Some OMPs in other bacterial pathogens are also reported to have a protective role against infection of pathogenic bacteria, such as *Vibrio parahaemolyticus* (e.g. Mao et al., 2007; Peng et al., 2016) and *Aeromonas hydrophila* (e.g. Dash, Sahoo, Gupta, Garg, & Dixit, 2014; Maiti, Shetty, Shekar, Karunasagar, & Karunasagar, 2012). It seems possible that OMPs in pathogenic bacteria can be used as vaccine candidate antigens but their protective effect may need an overall and comprehensive evaluation.

It is inevitable to mention that an unexpected additional band with similar molecular weight was detected when the recombinant protein, rOmep was produced. This additional protein was also reactive with sera of grass carp immunized with rOmep. One possible explanation for this is the production of truncated Omep by *E. coli* and a similar phenomenon was observed by Olivares-Fuster et al. (2010) where four protein bands were obtained when expressing DnaJ gene of *F. columnare* in a similar host system.

In conclusion, a total of five proteins were identified as immunogenic proteins in OMPs of *F. columnare* by employing an immuno-proteomic approach. The GldJ, Lip and Omep proteins demonstrated a good level of protection against *F. columnare* infection and may be considered as future candidate antigens for vaccine development against columnaris disease in freshwater fish.

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