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# Protection against *Vibrio alginolyticus* in pearl gentian grouper (♀*Epinephelus fuscoguttatus* × ♂*Epinephelus lanceolatu*) immunized with an *acfA*-deletion live attenuated vaccine

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ABSTRACT

*Vibrio alginolyticus* is well-known as an opportunistic Gram-negative pathogen, which endangers the development of global aquaculture as well as human health. In this study, a Δ*acfA* mutant strain and complementation of the Δ*acfA* mutant (C-*acfA*) were constructed. The Δ*acfA* mutant was tested in pearl gentian grouper (♀*Epinephelus fuscoguttatus* × ♂*Epinephelus lanceolatu*) to observe the changes in virulence and evaluate its potential as an attenuated live vaccine. The results showed that the Δ*acfA* mutant caused a high antibody titer and a significant reduction in the ability to colonize the intestine of pearl gentian grouper. Grouper vaccinated with Δ*acfA* mutant were more tolerant of the infection by virulent *V. alginolyticus* HY9901 without inducing clinical symptoms and obvious pathological changes. The relative percent survival value of pearl gentian grouper vaccinated with Δ*acfA* mutant intraperitoneal injection reached 81.1% after challenging with *V. alginolyticus* HY9901. The specific antibody titers immunized with Δ*acfA* was significantly higher than that in the PBS group. The antibody titer of Δ*acfA* group displayed the tendency of rising up from the first to fourth week and declining from fifth to eighth week and reached the peak at the fourth week. In the meanwhile, the expression level of genes associated with immunity, including IL-1β, TNF-α, IL-16, IgM, CD8α and MHC-Iα, was up-regulated after vaccination, indicating that the Δ*acfA* can induce effective and durable immune response in pearl gentian grouper and it may be an effective attenuated live vaccine candidate for the prevention of infections by *V. alginolyticus*.

## 1. Introduction

*Vibrio alginolyticus*, one of common pathogenic bacteria, can be found ubiquitously in marine and estuarine environment [1]. It is reported that *Vibrio alginolyticus* is closely related to some severe disease outbreaks in some marine commercial fishes in the coastal provinces of South China [2].

So far, there has been a lot of researches associated with virulence factors of *Vibrio alginolyticus*, such as hemolysin, extracellular product, lipopolysaccharide, iron carrier, extracellular product, tri-type secretory system and biofilm [3–8]. Besides, further studies have been contributed to some key steps of colonization of vibrio in the small intestine in the early stage in human, mouse and fish [9,10]. Pathogen selectively colonizes the surface of the small intestine, and then to grow and invade further with virulence factor, which causes damage to the tissue and induces the occurrence of diseases [9].

AcfA is one of accessory colonization factors (ACFs) that is being studied in the recent decades. Scholars suspect that *acfA* plays an significant role in efficient colonization of *Vibrio* in the intestine and have done some researches for controlling *Vibrio* disease [11,12]. Both *acfA* and *acfD* transcription are activated by *Vibrio cholerae* ToxT, which requires to bind to two centrally located DNA sites in an inverted repeat conformation [13]. Sharma et al. (2008) developed an efficient oral vaccine against cholera in transgenic tomato by combining *acfA* and cholera toxin b subunit [12]. Recently, a detailed study was performed on physiology and virulence of Δ*acfA* mutant, but it didn't reveal the potential of Δ*acfA* mutant as an attenuated vaccine candidate against infection caused by *V. alginolyticus* [11].

Compared with different kinds of chemical drugs, developing and applying vaccines to prevent major aquatic diseases is becoming an effective alternative for aquaculture industry. Because the vaccine not only improves the specific immunity of animal body, but it can also

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**Table 1**
Bacterial strains, plasmids, cell line and primers used in this study.

| Strain, Plasmid, Cell line and Primer | Relevant information or sequence (5'-3') | Source |
|---|---|---|
| **Bacterial Strains** | | |
| ***V. alginolyticus* HY9901** | Wild type strain | Cai et al., 2007a |
| **Δ*acfA*** | *V. alginolyticus* HY9901, in-frame deletion of $acfA_{61\text{-}548}$ | This study |
| **C-*acfA*** | HY9901Δ*acfA* containing plasmid of pBAD33 cm-*acfA* | This study |
| ***E. coli* DH5α** | Competent cells | TakaRa |
| ***E. coli* β2163** | Competent cells | Maibo Bioscience |
| **Plasmids** | | |
| **pLP12** | *E. coli*- suicide vector | This study |
| **pBAD33 cm-rp4** | *E. coli*- suicide vector | This study |
| **Primers** | Nucleotide sequence (5'-3') | |
| **AcfA-UF** | GGAATCTAGACCTTGAGTCGGGATTTTGTTACCAAGCGCCA | This study |
| **AcfA -UR** | CTATGGTGAGCTTTGGCAACACAGGAGCCGCAGATACCGAAA | This study |
| **AcfA-DF** | TTTCGGTATCTGCGGCTCCTGTGTTGCCAAAGCTCACCATAG | This study |
| **AcfA-DR** | ACAGCTAGCGACGATATGTCTGGAGAACCCTATCATTCGAGAAC | This study |
| **AcfA -TF** | CGCTTATCAACACCGTCCTTTAGA | This study |
| **AcfA -TR** | TGAGCTGCCTAAAGTAATCGACG | This study |
| **pBAD30-ZF** | CTAGAGTCGACCTGCAGGCA | This study |
| **pBAD30-ZR** | AGCTCGAATTCGCTAGCCCA | This study |
| **AcfA-RF** | TGGGCTAGCGAATTCGAGCTAGGAGGAATTCACCATGAACAAAACC | This study |
| **AcfA-RR** | TGCCTGCAGGTCGACTCTAGTTAGAAGTAATAAGTTGTGCC | This study |
| **PBAD-mcf-TF** | CCATAAGATTAGCGGATCCTACCT | This study |
| **PBAD-mcf-TR** | CTTCTCTCATCCGCCAAAACAG | This study |
| **IL-1βF** | TCTGGGCATCAAGGGCACACA | This study |
| **IL-1βR** | CCATGTCGCTGTTCGGATCGA | This study |
| **TNF-αF** | GCCACAGGATCTGGCGCTACTC | This study |
| **TNF-αR** | CTTCCGTCGCTGTCCTCATGTG | This study |
| **IL-16F** | TTCAGATCCTCCGTCCAAC | This study |
| **IL-16R** | TCTGTTCTGCGGGTTTAGC | This study |
| **IgMF** | TACAGCCTCTGGATTAGACATTAG | This study |
| **IgMR** | CTGCTGTCTGCTGTTGTCTGTGGAG | This study |
| **CD8αF** | GCTGGTGATTCTGCTGATTTG | This study |
| **CD8αR** | GGACTTGGAGGATGACTTTAGG | This study |
| **MHC-IαF** | GCCGCCACGCTACAGGTTTCTA | This study |
| **MHC-IαR** | TCCATCGTGGTTGGGGATGATC | This study |
| **β-actinF** | GGACAGCTACGTTGGTGATGA | This study |
| **β-actinR** | TGGTCACAATACCGTGCTCAATG | This study |

meet the requirements of non-polluting environment and non-drug-residue in aquatic products [14]. The attenuated live vaccine is a virulence-attenuated mutated pathogen prepared via various treatments, and it retains its immunogenicity. In addition, it will not cause the disease after being inoculated into the body, then the pathogen can grow and multiply within the body, induce the immune response, which may sustain a long-term or lifelong protection [15–17]. In this study, the attenuated live vaccine was prepared by in-frame knocking out *acfA* in *V. alginolyticus*.

In the present research, intestinal colonization of the Δ*acfA* mutant was observed and analyzed. Moreover, pearl gentian grouper was used to study the toxic effect of Δ*acfA* mutant and to evaluate potential of the mutant strain as an attenuated live vaccine via fish challenge, fish vaccination, antibody test, histopathological observation and qRT-PCR.

## 2. Materials and methods

### 2.1. Bacterial strains, fish and culture conditions

Bacterial strains and the plasmids used in this study are listed in Table 1. The *V. alginolyticus* HY9901 (WT) strain was isolated from diseased fish in Zhanjiang harbor area of Guangdong province, China, and it was cultured with Tryptic Soy Broth (TSB, Haling, China) at 28 °C. *Escherichia coli* DH5α was cultured with Luria-Bertani broth (LB, Haling, China).

Healthy pearl gentian grouper of similar body weight body weight (60 ± 2 g) were purchased from a fish farm in Dongnan Harbor (Guangdong Province, China). Fish are considered healthy by sera agglutination and bacteriological recovery tests described by Pang et al. [18]. The groupers were grown in 500 l containers with constant aeration and a temperature of 28 ± 1 °C. Fish were fed twice a day with a formulated pellet (No. 4c grouper feed, manufactured by Dongwan Feed Group, Zhanjiang, China). Pearl gentian grouper were acclimated to the indoor laboratory condition for 2 weeks before experiment. Groupers were anaesthetized with tricaine methane sulfonate (MS222) (Kuer, Anwei, China) before injections and sample collection. Animal experiments were complied with ethical standards and approved by Guangdong Provincial Key Laboratory of Pathogenic Biology and Epidemiology for Aquatic Economic Animals Ethics Committee.

### 2.2. Construction of a ΔacfA knockout mutant and complementation of the mutant

The Δ*acfA* mutant was constructed with allelic exchange mutagenesis. Firstly, two specific pairs of primers were designed to obtain the *acfA* upstream homologous arm fragment A (Primers: AcfA-UF and AcfA-UR) and downstream homologous arm fragment B (Primers: AcfA-DF and AcfA-DR). Then, fragment A and B are used as templates, and the AB fragments are fused together by overlapping PCR. The AB fusion fragment was connected to the suicide vector pLP12, and the recombinant product was transformed into the *E. coli* DH5α. The positive clone was selected and the recombinant plasmid plP12-AcfA was extracted. PLP12-*acfA* was transformed into *E. coli* β2163. The positive clones were selected and used for the conjugation with *V. alginolyticus*, and after twice homologous recombination, Δ*acfA* mutant was successfully constructed with a PCR identification (Primers: AcfA-TF and AcfA-TR).

The *acfA* fragment (Primers: AcfA-RF and AcfA-RR) and pBAD33 cm-rp4 vector fragment (Primers: pBAD30-ZF and pBAD30-ZR) were amplified, connected and then transformed into *E. coli* DH5α.

The positive clones were selected (Primers: pBAD-mcf-TF and pBAD-mcf-TR) and the recombinant plasmid was selected and transformed into *E. coli* β2163. The Δ*acfA* mutant strain conjugates with *E. coli* β2163. Positive clones were selected (Primers: pBAD-mcf-TF and pBAD-mcf-TR) and sequenced to confirm the successful construction of *acfA* complementation strain (C-*acfA*).

### 2.3. Pearl gentian grouper challenge

The challenge experiment was performed to determine the virulence of WT strain, Δ*acfA* mutant strain and C-*acfA* strain. The fish (n = 40) were anaesthetized by immersion in a 20 mg/l solution of MS-222 and then challenged with 100 μl $8.4 \times 10^8$ CFU/ml WT, 100 μl $8.1 \times 10^8$ CFU/ml Δ*acfA* and 100 μl $8.6 \times 10^8$ CFU/ml C-*acfA* by intraperitoneal injection, respectively. The cumulative mortality was monitored for 14 days post-challenge and the percentage survival was calculated. The experiment was duplicated triplicate. The bacteria were identified by re-isolating from the liver, kidney and spleen tissues of all dead fishes at the end of the experiment.

### 2.4. Pearl gentian grouper vaccination

Vaccination experiments were carried out to evaluate the potential of *ΔacfA* as an attenuated live vaccine candidate against *V. alginolyticus*. 80 pearl gentian grouper were divided into PBS group (control group) and Δ*acfA* group (the treated group). They were immunized intraperitoneally with 100 μl sterilized PBS and 100 μl $6.6 \times 10^5$ CFU/ml *ΔacfA* strain, respectively. After 28 days post-immunization, the pearl gentian grouper were challenged intraperitoneally with 100 μl $8.1 \times 10^8$ CFU/ml WT strain. The cumulative mortality was monitored for 14 days post-challenge. The experiment was duplicated triplicate. The relative percent survival (RPS) was calculated according to the following formula: RPS = [1 - (mortality of vaccinated fish/mortality of control fish)] × 100 [19].

### 2.5. Analysis of antibody levels

Serum of PBS group and Δ*acfA* group were collected to measure antibody levels using ELISA during the first to eighth week post-vaccination. The 96-hole ELISA plate was coated with 100 μl of formalin-killed *V. alginolyticus* by overnight incubation at 4 °C. Two-fold serial dilutions of the fish serum samples were added to the 96-hole plate blocked with 2% BSA. Antibody binding to the antigen was detected using *Epinephelus coioides* IgM monoclonal antibody. Plates were incubated with rabbit anti-mouse IgG-HRP conjugates. The reaction was developed with the 3,3,5,5-tetramethylbenzidine (TMB) substrate with $H_2O_2$ and stopped with 2 M $H_2SO_4$. Optical density was measured at 450 nm using a micro plate reader.

### 2.6. Colonization ability of the different V. alginolyticus strains in the intestine

This assay was performed based on the study of Whitaker et al. with some modification [20]. 90 pearl gentian grouper were divided into WT group, Δ*acfA* group and C-*acfA* group. Fish were orally dosed with 1 ml $3.2 \times 10^7$ WT strain, 1 ml $3.1 \times 10^7$ CFU/ml Δ*acfA* strain and 1 ml $3.4 \times 10^7$ CFU/ml C-*acfA* strain. After 48 h post-challenge, intestine was removed aseptically from the WT group, the Δ*acfA* group and C-*acfA* group. The experiment was duplicated triplicate. For total intestinal CFU determination, the entire intestine was sampled and washed with PBS to remove the fecal contents, Intestinal mucosa was collected and then homogenized in 1 ml of PBS. Homogenized samples were serially diluted, plated on TCBS mediun, and incubated at 28 °C.

### 2.7. Histopathological sections post-vaccination

After 28 d post-immunization, in order to evaluate the safety of attenuated Δ*acfA*, the spleens and livers were collected from the PBS group and the Δ*acfA* group and used to perform histological examination. Samples of WT group of challenge experiment (2.3) were used as pathogen positive control. Spleens and livers tissues were fixed in Davidson's Fixative (Shanghai Tarui Bioscience) for 24 h. Then the tissues were orderly dehydrated in ethanol with different concentrations (50%→70%→80%→90%→95%→100%), equilibrated with xylene, and embedded in paraffin. Paraffin block were then cut to 5 μm thickness, stained by Haematoxylin–Eosine and examined for pathological alterations with an optical microscope.

### 2.8. Immune-related gene expression analysis

Mesonephros samples were collected at various times for Real-time PCR to examine the alteration of immune-related gene expression during the 28 d-vaccination. Real-time PCR was performed in triplicate according to standard protocols with the use of SYBR® Green qPCR superMix kit (TransGen, China). The final reaction volume of 20 μl contained 1 μl cDNA, 10 μl of 2 × SYBR Green qPCR superMix, 1 μl of each primer (10 μM), and 7 μl of ddH2O. The reaction was performed under the following conditions: 95 °C, 4 min; 95 °C, 20 s; 60 °C, 30 s; 72 °C, 30 s (40 cycles). A melting curve analysis was performed to access amplification of specific products. The β-actin gene was used as an endogenous control. The IL-1β, TNF-α, IL-16, IgM, CD8α and MHC-Iα gene expression were normalized to β-actin and reported as relative expression values using the $2^{-\Delta\Delta Ct}$ method [21].

### 2.9. Statistical analysis

The results were examined with a one - way analysis of variance (ANOVA) using the SPSS 19.0 computer program (SPSS Inc., Chicago, USA). Mean comparisons were tested using Duncan's test. Data are presented as mean ± SE and subjected to Student's t - test for determining significant differences between groups. The differences were considered significant if $p < 0.05$.

## 3. Results

### 3.1. Identification of the ΔacfA mutant

An isogenic *acfA* mutant of the wild strain HY9901 was constructed through allelic exchange mutagenesis. The result was confirmed by a PCR identification (Fig. 1) and direct DNA sequencing (data not shown), verifying that a knockout mutant of Δ*acfA* was successfully constructed without alteration of the remaining sequences.

### 3.2. Challenge and virulence of V. alginolyticus ΔacfA mutant strain

The challenge experiment was used to compare virulence of *V.*

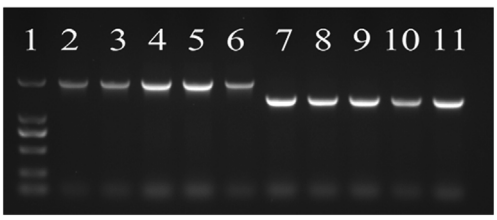


**Fig. 1.** PCR identification of *V. alginolyticus* strain Δ*acfA* (Primers: AcfA-TF and AcfA-TR). 1: 2, 000 DNA marker; 2–6: PCR amplifications with wild type HY9901 genomic DNA (1887bp); 7–11: PCR amplifications with Δ*acfA* genomic DNA (1398bp).

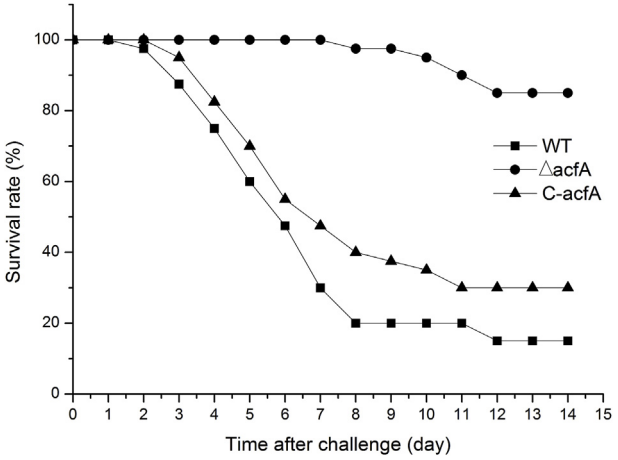


**Fig. 2.** Survival rate of grouper after challenge with WT (■), Δ*acfA* (●) and C-*acfA* (▲).

*alginolyticus* WT strain, Δ*acfA* strain and C-*acfA* strain. The result showed that the grouper survival rate in the Δ*acfA* group and C-*acfA* group was significantly higher than that in the WT group ($p < 0.01$) (Fig. 2). In the WT group and C-*acfA* group, the fish began to die on the third day, resulting in the cumulative mortalities were 85% and 70%, whereas only 15% of the fish challenge with Δ*acfA* mutant were dead in the challenge experiment. The results indicated that *V. alginolyticus* Δ*acfA* was lowly virulent and safe to pearl gentian grouper and had the potential as an attenuated live vaccine.

### 3.3. Immunoprotection of ΔacfA strain in pearl gentian grouper

The immune protective efficacy of Δ*acfA* was evaluated in this experiment. Grouper were challenged with *V. alginolyticus* WT strain at 28 d after vaccinating with PBS and Δ*acfA* strain. Fig. 3 showed that the survival rate in the Δ*acfA* group was 82.5% with a RPS of 81.1% ($p < 0.01$), which was significantly lower than that of PBS group.

### 3.4. Histological analysis of liver and spleen post-immunization

There was no mortality during the whole immunization experiment. Compared with PBS group, fish immunized Δ*acfA* did not exhibit obvious symptoms of hemorrhaging, septicemia, dark skin, and ulcers on the skin surface, and sample of Δ*acfA* group showed no histologically abnormality. However, distinct histological changes were observed in the samples of WT group. In liver: hemorrhage, enlargement, the nucleus enlarged and the cytoplasmic ratio reduced obviously; In spleen: hemorrhage, enlargement, inflammation and splenic cord were unclear, the red pulp area increased (Fig. 4).

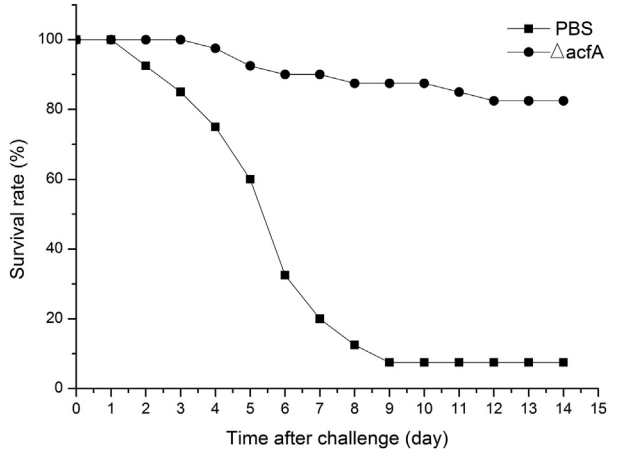


**Fig. 3.** Survival rate of grouper after challenge with *V. alginolyticus* WT. Fish was vaccinated with Δ*acfA* (●). Control fish was injected with PBS (■).

### 3.5. Antibody levels

The antibody level of grouper immunized with PBS (control) and Δ*acfA* strain was assessed by ELISA during the first to eighth week after vaccination(Fig. 5). The result showed that antibodies in the immunized group could be detected at the first week after vaccination. The specific antibody titers immunized with Δ*acfA* was significantly higher than that in the PBS group ($p < 0.05$), the antibody titer of Δ*acfA* group displayed the tendency of rising up during the first to fourth week and declining during the fifth to eighth week and reached the peak at the fourth week ($p < 0.01$).

### 3.6. Colonization ability of the different V. alginolyticus strains in the intestine

The assay was performed to detect the colonization ability of the different *V. alginolyticus* strains in the intestine (Fig. 6). The data showed that *V. alginolyticus* Δ*acfA* CFU significantly decrease by 11.9 times and 11.2 times when compared with WT group and C-*acfA* group.

### 3.7. Expression of immune-related genes during immunization

During the immunization phase, qRT-PCR was performed to analyze the transcription level of immune-related genes (IL-1β, TNF-α, IL-16, IgM, CD8α and MHC-Iα) during the 28-d immunization (Fig. 7). The immune-related gene, IL-1β, TNF-α, IL-16, IgM, CD8α and MHC-Iα, were significantly activated after Δ*acfA* mutant immunization. The IL-1β expression level significantly increased at 24 h (7.1-folds) and in 7th day (14.2-folds). Similar changes were observed in TNF-α (5.3-folds at 6 h and 8.8-folds in 7th day), IL-16 (4.3-folds at 12 h, 4.2-folds at 24 h and 3.5-folds in 7th day), IgM (7.9-folds at 24 h and 6.6-folds in 14th day). In the meantime, MHC-Iα and CD8α reached their peaks at 24 h (8.9-folds) and in 2th day (4.1-folds) following immunization.

## 4. Discussion

Colonization is a microecological manifestation of the symbiotic relationship between normal microorganism and host in the long history of evolution, interrelating with physiological status of microorganism and the capacity of host to resist adhesion. The *acfA* gene, one of accessory colonization factors, is closely associated with polar flagellum forming, motility, and biofilm formation, while playing an important role in regulating the normal function of secretion systems for virulence proteins and bacterial virulence in *V. alginolyticus* [11]. In addition, previous research suggested it enables the pathogen to adhere to the host intestinal mucosa without being effectively cleared by intestinal peristalsis and intestinal secretion [13]. In the present study, Δ*acfA* mutant strain was defective in colonization compared to WT and C-*acfA* strain. Decrease in motility and suppression of polar flagellum forming caused by the *acfA* gene mutation may well account for its weaken ability to colonize in intestines compared with wild type stain [11]. Similar result can be seen in the work of Peterson et al. that after knockout of the *acfA* gene of *Vbrio cholerae*, the pathogenic bacteria reduced the effective colonization ability in the intestinal tract of mice, and significantly cripple the ability to infect the mice [22].

To our knowledge, there is no commercial vaccine targeting on to vibriosis in China, although the disease has been causing great economic loss to aquaculture industry [23,24]. Since *acfA* makes a great difference to regulation for pathogenesis of fish pathogen *V. alginolyticus*, various vaccines aiming at *acfA* have been developed in recent years, such as DNA vaccine and recombinant ACFA, both of the vaccines have acquired certain effects, the RPS of them were between 73%

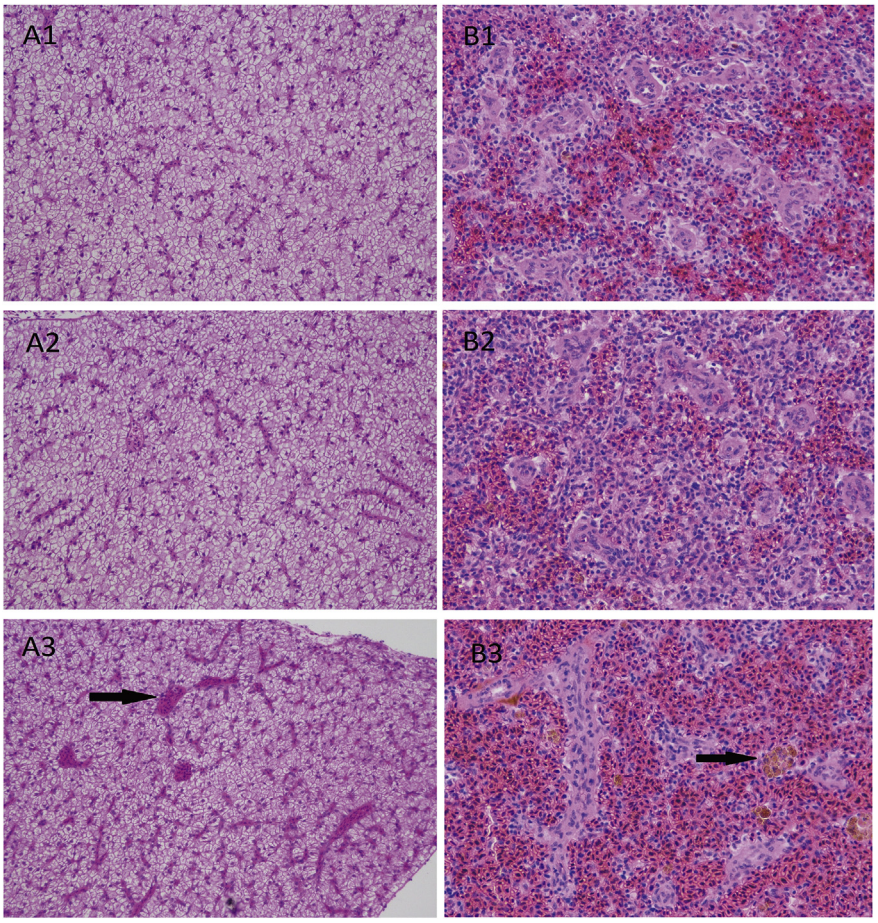


**Fig. 4.** Histological changes of liver (A) (200 × magnification) and spleen (B) (400 × magnification) tissues stained with Hematoxylin - Eosin. (A1, B1), PBS group; (A2, B2), Δ*acfA* group and (A3, B3), WT group. (A3) Liver of WT infected fish show hemorrhage (→), enlargement, the nucleus enlarged and the cytoplasmic ratio reduced obviously. (B3) Spleen of WT infected fish show hemorrhage, enlargement, inflammation, an increasement in lymphocytes (→), splenic cord were unclear, the red pulp area increased. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

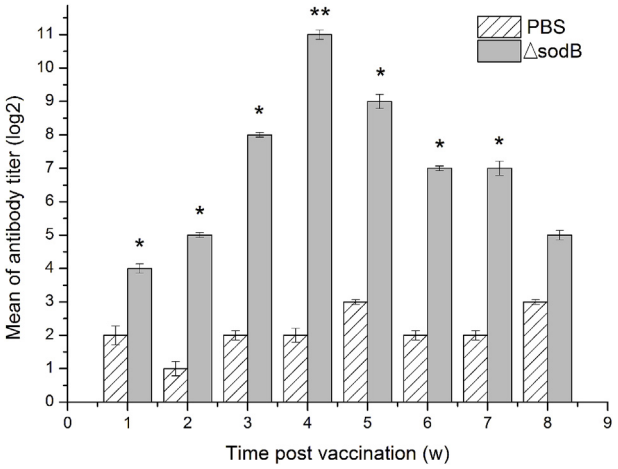


**Fig. 5.** Analysis of antibody levels of grouper immunized with PBS and Δ*acfA* strain by ELISA. Sera samples were collected at week 1–8 after vaccination. Each datum column represented the mean of log 2 antibody titers with standard error bar. The asterisks indicated significant differences (*$p < 0.05$, **$p < 0.01$) between the control and the immunized group.

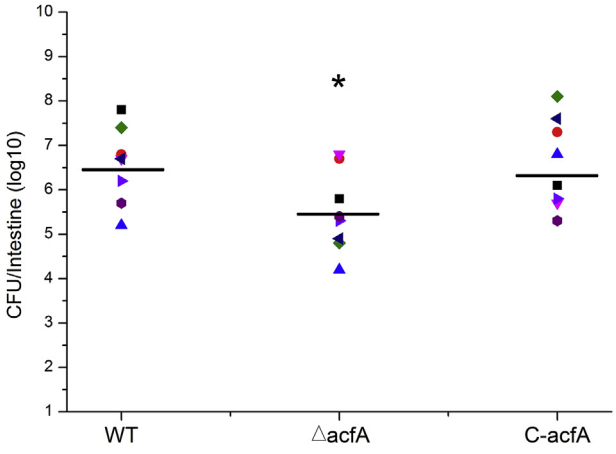


**Fig. 6.** Intestine colonization of *V. alginolyticus* WT, Δ*acfA* strain and C-*acfA*. Grouper were orally dosed with 1 ml 3.2 × $10^7$ WT strain, 1 ml 3.1 × $10^7$ CFU/ml Δ*acfA* strain and 1 ml 3.4 × $10^7$ CFU/ml C-*acfA* strain. At 48 h post-infection, the entire small intestine were collected from infected fish. The asterisks indicated significant differences (*$p < 0.05$) among the groups.

and 96% [10,25]. Currently, virulence genes deletion is becoming a common strategy for preparing live attenuated vaccine, which outstands for low toxicity and sustainable protection without causing vaccinated animals a disease. In the recent years, it exhibits its great potential in preventing and controlling some hazardous bacterial diseases [18,26,27]. DNA vaccine, subunit vaccine, live attenuated vaccine and other vaccines makes a significant contribution to the development of vaccine against vibriosis. Protection effect could be varied among these vaccines. The differences of protection effect may be caused by different sequence of amino acids, different virulence in bacterium, different immune tolerance in the tested animals, different bacterial dose and inoculation method in the challenge experiment [28]. However, a major concern for safety and endurance is the potential risk of mutated bacteria - the possibility of restoring toxicity in the body due to retrograde mutations. Several experiments in this study were carried out evaluate the potential of Δ*acfA* mutant as a live attenuated vaccine.

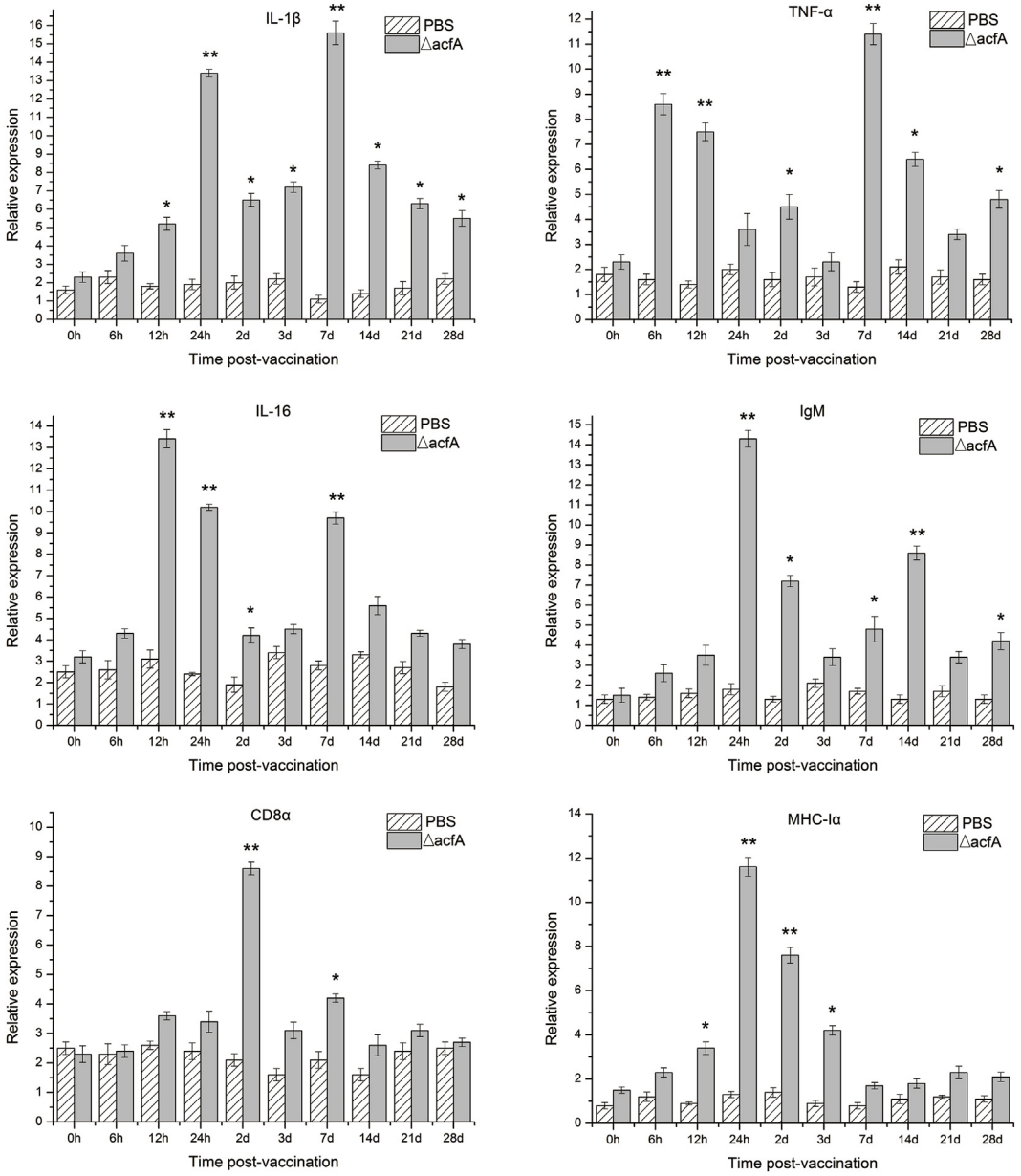


**Fig. 7.** The expression of immune-related genes in spleen of pearl gentian grouper by qRT-PCR during immunization. The mRNA level of each immune-related gene was normalized to that of β-actin and relative expression was calculated by deciding the values of the vaccinated tissues by those of the controls. Bars represented the mean relative expression of three biological replicates and error bars represented standard error. **: $p < 0.01$, *: $p < 0.5$.

In the challenge experiment, the data showed the cumulative mortalities of Δ*acfA* group was significantly lower in contrast to WT group and C-*acfA* group, which indicated that Δ*acfA* mutant was markedly attenuated in toxicity and infectivity in pearl gentian grouper. Moreover, In *V. alginolyticus*-challenged experiment, the RPS of pearl gentian grouper vaccinated with Δ*acfA* mutant reached 81.1%, it was significantly higher than that of the control group and C-*acfA* group ($p < 0.01$). Some previous studies have also demonstrated that valid protective immunity can be induced by knock-out mutants. For example, the RPS of Sortase A (srtA) mutant strain of *Streptococcus iniae* was as high as 95.5% in Nile tilapia [27]. Choi et al. reported that *Δalr1Δalr2* mutant strain of *V. anguillarum* provided powerful protection for *Paralichthys olivaceus* and showed no mortality [29]. The results showed *acfA* mutant could provide effective protection challenge with virulent *V. alginolyti*cus. In addition, vaccination with *ΔacfA* mutant could also increase antibody levels significantly against pathogenic *V. alginolyticus*, Similar result has been reported in *V. alginolyticus* by knocking out the *Vsco* gene [28]. The result suggested Δ*acfA* mutant could induced a powerful and long-lasting B cell-mediated humoral immune response.

The spleen is the hemopoietic tissue as well as an important peripheral immune organ to fish. Large numbers of macrophages in the spleen can clear aging blood cells (such as red blood cells), antigens and foreign bodies [30]. IL-1β stimulates the production of cytokines such as colony stimulating factor, platelet growth factor and enable T cells to produce interleukin-2, which plays a role in immune response and tissue repair [31]. TNF-α, a monokine produced mainly by monocyte-phagocyte, which helps the body resist infection with a variety of immunomodulatory effects [32]. As an important T cell chemotactic factor, IL-16 is widely involved in inflammatory response and immune regulation [33]. In this study, IL-1β, TNF-α and IL-16 were upregulated in different time-points post-vaccination in a similar pattern, suggesting innate immune response could be induced by Δ*acfA* mutant. Besides, expression changes in specific immune associated genes, such as IgM,

CD8α and MHC-Iα, were also analyzed. IgM, with the participation of complements and phagocytes, is the "pioneer" in the body's fight against infection by killing bacteria, lysing bacteria and activating complements to promote phagocytosis [34]. CD8 is a subset of T lymphocytes and plays an important role in the recognition and presentation of antigens in specific immune responses. CD8 binds to MHC I molecules of other immune cells, such as CD4, by binding to MHC II molecules on the surface of CD8, thus recognizing antigens bound to other immune cells. In the present study, IgM, CD8α and MHC-Iα were significantly elevated in the start and middle time-points of the experiment. The profile of immune responses in these genes was in line with the study which carried out on *Trachinotus ovatus* and *Danio rerio* with live attenuated *S. agalactiae* and *V. anguillarum* [19,35]. The results showed that the expression of immune-related genes can be greatly induced by immunization with Δ*acfA* mutant in pearl gentian grouper and thus played an important role in defending against pathogenic *V. alginolyticus*.

## 5. Conclusion

In conclusion, our findings indicated that the colonization ability and virulence of *Vibrio alginolyticus* HY9901 was weaken by *acfA* deletion. Moreover, the Δ*acfA* mutant can provide high protection efficacy against virulent homologous challenge with a high antibody titer and activated immune - related genes, and effectively induce a powerful and long-lasting B cell-mediated humoral immune response without causing clinical symptoms and obvious pathological changes in pearl gentian grouper. The present study may contribute to the further development of live attenuated vaccine for preventing and controlling bacterial infection.

## Conflicts of interest

The authors declare no conflict of interest.

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