



Evaluation of Immunogenicity, Protective Immunity on Aquaculture Pathogenic *Vibrio* and Fermentation of *Vibrio alginolyticus* Flagellin FlaC Protein

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Abstract

Background: *Vibrio* are the main pathogenic bacteria in aquaculture. The flagellin protein C (FlaC) of *Vibrio alginolyticus* has good immunogenicity and the prospect of potential application in a vaccine.

Objectives: We aimed to evaluate the immunogenicity, protective immunity, and prokaryotic expression fermentation of *V. alginolyticus* FlaC protein for the vaccine in aquaculture.

Material and Methods: A molecular cloning method was used to construct the expression strain of FlaC protein, and the protein was purified with Ni-affinity chromatography. Polyclonal antiserum was prepared via mice immunized with the FlaC protein. The Western blot and enzyme-linked immunosorbent assay (ELISA) were used to check the specificity and titre of the antiserum. ELISA and pull-down assay detected the interaction between FlaC protein antiserum and *Vibrio*. The immune protection function of FlaC protein was detected with mice actively immunized with FlaC protein and challenged by *V. alginolyticus* and *V. parahaemolyticus*. The optimal expression conditions for FlaC protein were detected using an L₉(3⁴) orthogonal design model.

Results: The expression strain of FlaC protein was obtained successfully, and purified FlaC protein was prepared using a mice polyclonal antibody. The FlaC protein antiserum held a high specificity, and the titre was 1:3200. The antiserum directly interacted with *V. alginolyticus* and *V. parahaemolyticus*, and the FlaC protein demonstrated a significant immune protection function (50%) against *V. alginolyticus* infection and some immune protection function (41.66%) against *V. parahaemolyticus*. The optimal expression conditions for FlaC protein included a strain OD₆₀₀ value of 0.8, final isopropyl-β-d-thiogalactoside (IPTG) concentration of 0.1 mmol/L, an inducing time of 8 hours, and an inducing temperature of 28°C.

Conclusions: This study showed that the FlaC protein possesses a significant immunogenicity and immune protection effect and obtained the optimal fermentation conditions. It is expected to be a potential vaccine against *V. alginolyticus* and *V. parahaemolyticus*.

Keywords: *Vibrio alginolyticus*; *Vibrio parahaemolyticus*; FlaJ Protein; *Vibrio parahaemolyticus*

1. Background

Vibrio alginolyticus and *Vibrio parahaemolyticus* are major aquaculture pathogenic *Vibrio* (1),

distributed in sea areas and estuaries, which causes septicemia and gastroenteritis in fish and shrimp

(2), induces a large number of deaths, and affects the development of aquaculture (3). Pathogens coinfecting humans and marine animals, *V. alginolyticus* and *V. parahaemolyticus* can also cause gastrointestinal diseases and wound infections in humans (4). At present, antibiotics are mainly used in the prevention of animal diseases and the treatment of *Vibrio* infection (5). This usage inevitably leads to drug residues, bacterial resistance, and environmental pollution (6). Therefore, it is necessary to develop new drug preparations.

Vibrio usually has three kinds of antigens, which are flagellum antigen, bacterium antigen, and surface antigen (7). These antigens provide the interaction between bacteria and host and are a prerequisite for the preparation of vaccine with strong immune protection specificity. *Vibrio* flagella are essential components in the pathogenic potential of bacteria, because they enhance the motility and adhesion ability of the bacteria (7). They are also important bacterial surface antigens, which are closely related to bacterial immune protection (8). Flagellin protein C (FlaC), FlaB, and FlaI of *Vibrio* can activate the immune protection function of animals, and they have good immunogenicity and hold the prospect of potential application in vaccines. FlaC protein is a granular protein, the main protein responsible for the flagellum fibres of *V. alginolyticus*, providing the basis for the movement of the bacteria (9). It has been found to have good immunogenicity, which activates the immune system of animals and enhances the immune protection function of *Lutjanus sanguineus* against *V. alginolyticus* (10). Thus, FlaC protein has a good prospect of application in a vaccine against *Vibrio* infection. It is, therefore, necessary to further research into the immune protection function and fermentation conditions of FlaC protein.

2. Objectives

In this study, we aimed to evaluate the immunogenicity, protective immunity, and prokaryotic expression fermentation of *V. alginolyticus* FlaC protein for the vaccine in aquaculture.

3. Materials and Methods

3.1. Ethics statement

The animal ethical committee (Ref. no20161120) was approved by the ethics committee of Shaanxi University of Technology, China.

3.2. Materials

V. alginolyticus, *V. parahaemolyticus*, the *Escherichia coli* DH5a strain, the *E. coli* BL21 strain, and pET-32a plasmid were obtained from the Bacterial Conservation Centre, China. Primer synthesis and gene sequencing

were completed by the Beijing Oak Science and Technology Corp., China. The mice were obtained from the College of Medicine, Xian Jiaotong University, China.

3.3. Cloning of FlaC Gene

The *flaC* gene primers were designed according to the *flaC* gene sequence of *V. alginolyticus* (GenBank database accession number FJ617268), including sense primer 5'-TAAGGATCCATGGCTGTAACAGTTAGT-3' and anti-sense primer 5'-CCTCTCGAGTTACTGCAATAGTGACAT-3',

where the first and second underscores represent the sites of the *Bam*H I and *Xho* I enzyme cleavages, respectively. The *V. alginolyticus* genome was extracted using a genomic extraction kit (TaKaRa, Japan). The polymerase chain reaction (PCR) system consisted of 2.5 μ L buffer, 2 μ L dNTP (10 mmol/L), 1.5 μ L primers (20 μ mol/L), 4 μ L template DNA, and 0.2 μ L Taq enzyme (TaKaRa, Japan). The PCR cycle parameters were pre-denatured for 2 minutes at 94°C and were then denatured, annealed, and extended for 32 cycles (30 s at 94°C, 45 s at 55°C, and 90 s at 72°C) and fully extended for 10 minutes at 72°C. PCR samples were separated and recovered by 0.8% agarose gel electrophoresis. After the PCR product and pET-32a plasmid vector were digested, the recombinant plasmid pET32a-*flaC* was obtained by ligase ligation (TaKaRa, Japan). The recombinant plasmid was identified through double-enzyme digestion and sequencing test. Then, it was transformed into an *E. coli* BL21 strain to construct the expression strain of FlaC protein.

3.4. Expression Detection and Purification of FlaC Protein

The expression and purification were implemented as described above. The *flaC* gene recombinant strains were cultured overnight and transferred to fresh LB medium. The OD₆₀₀ value was about 0.5, and the final concentration of 0.5 mmol/L isopropyl- β -D-thiogalactoside (IPTG) was added and cultured at 37°C for 5 hours. FlaC protein expression was detected by SDS-PAGE (11). The FlaC strain was loaded using Ni-affinity chromatography and purified using the Ni-NTA flow resin method (TaKaRa, Japan).

3.5. Preparation of Mouse Polyclonal Antiserum against FlaC Protein

Kunming mice were randomly selected. The experimental and control groups were immunized with FlaC protein (50 μ g per mouse) and phosphate-buffered saline (PBS) solution, respectively. Freund's complete adjuvant (Sigma, USA) was used for the first immunization. The second immunization followed after 14 days, this time using Freund's incomplete adjuvant (Sigma, USA). Seven days later, the third immunization was performed. Then the eyeballs of the mice were

removed under anaesthesia to obtain FlaC protein antiserum and stored in a refrigerator at -80°C .

3.6. Specificity and Titre Detection of FlaC Protein Antiserum

The specificity of FlaC protein antiserum was determined by the Western blot. Through SDS-PAGE, *V. alginolyticus* protein was transferred to a nitrocellulose (NC) membrane (TaKaRa, Japan). After incubating in skim milk solution for blocking, the membrane was incubated with mouse anti-FlaC protein antiserum and was then added to anti-mouse horseradish antibodies (TaKaRa, Japan). A DAB solution (Sigma, USA) colouration system was used on the NC membrane for band visualization. The FlaC protein serum specificity was determined according to the colour of the NC membrane bands (11).

The titre of the antiserum was detected using the enzyme-linked immunosorbent assay (ELISA). The FlaC protein was dissolved to $0.5\ \mu\text{g}/\mu\text{L}$, and $100\ \mu\text{L}$ of the solution was added to the 96-well plate at 37°C for 3 hours. After incubating in a skim milk solution for blocking, $100\ \mu\text{L}$ of anti-FlaC protein antiserum was added to each well at 37°C for 30 minutes. After rinsing, $100\ \mu\text{L}$ anti-mouse horseradish antibodies were added to each well. Then, a colouration solution (Sigma, USA) was added to every well at 37°C to prevent light from affecting the colour reaction. Finally, a stop solution was added, and a microplate reader (Thermo, USA) was used to detect the OD_{450} value (11).

3.7. Interaction between FlaC Protein Antiserum, *V. alginolyticus*, and *V. parahaemolyticus* by ELISA and Pull-down Assay

ELISA and pull-down assay were implemented as described. *V. alginolyticus* and *V. parahaemolyticus* were collected at the logarithmic growth phase by centrifuge and washed twice with NaCl solution (0.85%). The bacteria were deactivated and immobilized with oxymethylene at 80°C for 90 minutes. Then the samples were dissolved in 0.85% NaCl and adjusted to 0.2 at OD_{600} nm. Samples of 1 mL were transferred into 1.5-mL tubes with 10^8 CFU bacterial cells. Then $100\ \mu\text{L}$ FlaC protein antiserum was added to each tube, with $1.5\ \mu\text{g}/\mu\text{L}$ protein of bovine serum albumin used for the negative control. After rinsing, $100\ \mu\text{L}$ anti-mouse horseradish antiserum was also added to each tube. Colouration liquid was added to every tube to prevent a light reaction. After the stop solution was added to each tube, a microplate reader was used to detect the absorbance value at OD_{450} nm (11).

3.8. Immune Protection Function of FlaC Protein

Kunming mice were divided between an experimental group and a control group. Purified FlaC protein ($50\ \mu\text{g}$ per mouse) was injected three times and the control group was injected with PBS solution. The primary immunization was conducted with Freund's complete adjuvant, and booster doses with Freund's incomplete

adjuvant. After the third immunization, the mice were challenged with *V. alginolyticus* and *V. parahaemolyticus*. After 15 days, the relative percentage survival of the mice was measured, and the immune protection rates were expressed as formula of $1 - (\text{FlaC immunity mortality}/\text{non-protein immunity mortality}) \times 100\%$ (12). The Statistical Package for the Social Science (SPSS) software was used for significance analysis.

3.9. Induced Expression Condition of FlaC Protein

The $\text{L}_9(3^4)$ orthogonal design model—a four-factor, three-level orthogonal design—was used to detect the optimal expression conditions of FlaC protein. The factors of the orthogonal design included the strain of OD_{600} value, IPTG final concentration, inducing time, and inducing temperature, represented as A, B, C, and D, respectively (Table 2). In accordance with the orthogonal design model, when the OD_{600} concentration of FlaC protein expression of the bacteria was reached, corresponding concentrations of IPTG were added to the culture to induce FlaC protein expression at an appropriate time and temperature. One millilitre of bacterial liquid was harvested and boiled for 5 minutes with $300\ \mu\text{L}$ buffer solution. After centrifuge, $10\ \mu\text{L}$ samples were added for SDS-PAGE. G-250 dye liquor (Sigma, USA) was used to visualize the FlaC protein bands. Finally, Phoretix 1D software was used to analyse the optical density of the FlaC protein bands, and SPSS software was used to analyse the significance for each factor.

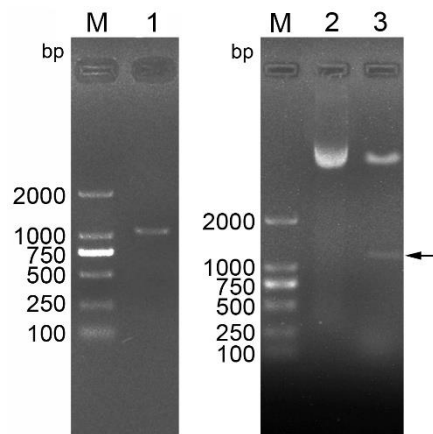


Figure 1. The polymerase chain reaction (PCR) and restriction enzyme analysis of *Vibrio alginolyticus* *flaC* gene. M, DNA marker; 1, the PCR of *flaC* gene; 2, the recombinant plasmid of *flaC* gene; 3, the recombinant plasmid digested by *Bam*HI and *Xho*I. Using PCR, a fragment of about 1155 bp was amplified from the genome of *V. alginolyticus*, which was consistent with the size of the *flaC* gene. The size of the double-enzyme digestion was consistent with the prediction recombinant gene.

4. Results

4.1. Construction of FlaC Gene Prokaryotic Expression Strain

A fragment of about 1155 bp was amplified from the genome of *V. alginolyticus* using PCR; this was

consistent with the expected size (Fig. 1). The target gene obtained by PCR was linked with pET-32a plasmid. The size of the target gene obtained by the double-enzyme digestion was consistent with the prediction (Fig. 1). Sequencing results also confirmed that the target gene was the same as the *flaC* gene sequence published by the National Center for Biotechnology Information database. Finally, *flaC* gene recombinant plasmid was transformed into the *E. coli* BL-21 strain to obtain the expression strain of FlaC protein.

4.2. Expression Detection and Purification of FlaC Protein

To verify the expression of the recombinant target protein, the bacteria containing the recombinant plasmid were induced with IPTG, and a protein band with a molecular weight of ~62 kDa was obtained, containing ~42.35 kDa of FlaC protein and 20.4 kDa fusion protein of pET-32a plasmid, which was consistent with the expected weight (Fig. 2). The purified FlaC protein was obtained using Ni-affinity chromatography (Fig. 2).

4.3. Specificity and Titre of FlaC Protein Antiserum

The Western blot was used to detect the specificity of the FlaC mice antiserum, and it detected a single band of FlaC protein, indicating that the FlaC protein antiserum had good specificity (Fig. 3A). The antiserum titre of FlaC protein reached 1:3200 with ELISA (Fig. 3B).

4.4. Interaction between FlaC Protein Antiserum, *V. alginolyticus*, and *V. parahaemolyticus*

The interaction between FlaC protein antiserum and *Vibrio* was detected using ELISA and pull-down assay. There was interaction between FlaC protein antiserum and *V. alginolyticus* until the 1:500 serum titre, and FlaC protein antiserum and *V. parahaemolyticus* until the 1:400 serum titre (Fig. 4). FlaC protein antiserum and major aquaculture pathogenic *Vibrio* formed antigen-antibody complexes, which probably provided for antigen presentation. It was revealed that FlaC protein may have a preferable immunogenicity.

4.5. Immunological Protection of FlaC Protein

Mice were immunized with FlaC protein and challenged with *V. alginolyticus* and *V. parahaemolyticus* to detect

the immunological protection of FlaC protein. The mice developed severe toxic symptoms, such as fluffy folds, sluggish activity, listlessness, and lethargy, and a large number died within 48 hours. After 4 days, death was controlled, and the mice gradually recovered. The FlaC protein demonstrated a significant immune protection function (50%) against *V. alginolyticus* infection and some immune protection function (41.66%) against *V. parahaemolyticus* (Table 1).

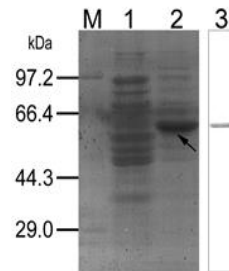


Figure 2. Expression detection and protein purification of flagellin protein C (FlaC). M, protein marker; 1, no inducing with isopropyl- β -D-thiogalactoside (IPTG); 2, inducing with IPTG; 3, purification of FlaC protein. The recombinant protein bacteria were induced with IPTG, obtaining a protein band with a molecular weight of ~62 kDa, containing ~42.35 kDa of FlaC protein and 20.4 kDa of fusion protein, which was consistent with the expected weight. The purified FlaC protein was obtained with Ni-affinity chromatography and showed one band.

4.6. Optimization of Prokaryotic Expression Conditions of FlaC Protein

In order to detect FlaC protein expression, orthogonal design experiments were conducted. The expression map of FlaC protein was obtained by SDS-PAGE, which showed that the FlaC protein expression amount differed under different induction conditions (Fig. 5). The optical density value of the FlaC protein band was obtained by Phoretix 1D software, and range analysis was conducted (Table 2). Comparing K1, K2, and K3, the optimal expression condition of FlaC protein was A2, B1, C2, and D1, which meant a strain OD₆₀₀ value of 0.8, a final IPTG concentration of 0.1 mmol/L, an induction time of 8 hours, and an induction temperature of 28°C. The variance analysis of the optical density data showed that four factors reached significance, including the strain OD₆₀₀ value, final IPTG concentration, induction time, and induction temperature (Table 3).

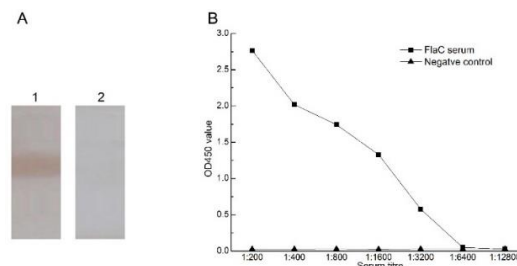


Figure 3. Specificity and titre of flagellin protein C (FlaC) antiserum. A and B show the specificity and titre, respectively, of the FlaC protein antiserum. 1 and 2 show the FlaC protein antiserum and negative control, respectively. 1 shows that one band was visualized, indicating that the FlaC protein antiserum had good specificity. As the FlaC protein antiserum titre increased, the OD₄₅₀ value decreased; the FlaC protein antiserum titre was 1:3200.

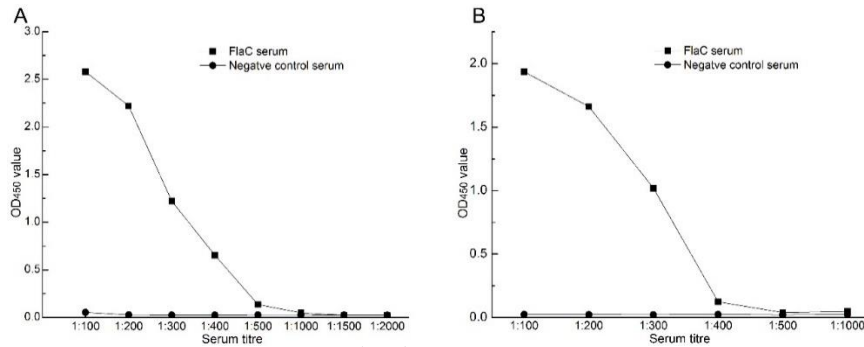


Figure 4. Analysis of the interaction between flagellin protein C (FlaC) antiserum, and *Vibrio alginolyticus*, *Vibrio parahaemolyticus*. A and B show the interaction between FlaC protein antiserum and *V. alginolyticus*, *V. parahaemolyticus*. As the FlaC protein antiserum titre increased, the OD_{450} value decreased. The interaction between FlaC protein antiserum and *V. alginolyticus* can be found until the 1:500 serum titre, and FlaC protein antiserum and *V. parahaemolyticus* until the 1:400 serum titre.

Table 1. Active Immunity FlaC and Challenging with *Vibrio alginolyticus* and *Vibrio parahaemolyticus* in Mice

Bacterial Name	FlaC				Control			
	Nos	Alive	ADR (%)	RPS (%)	Nos	Alive	ADR (%)	RPS (%)
<i>V. alginolyticus</i>	15	9	40	50*	15	3	80	—
<i>V. parahaemolyticus</i>	15	8	46.67	41.66	15	3	80	—

ADR, accumulating death rates; FlaC, flagellin protein C; RPS, relative percent survivals

RPS (%) = $1 - (\text{FlaC immunity mortality} / \text{non-protein immunity mortality}) \times 100\%$.

* $P < 0.05$ (compared to the control group which received phosphate-buffered saline only)

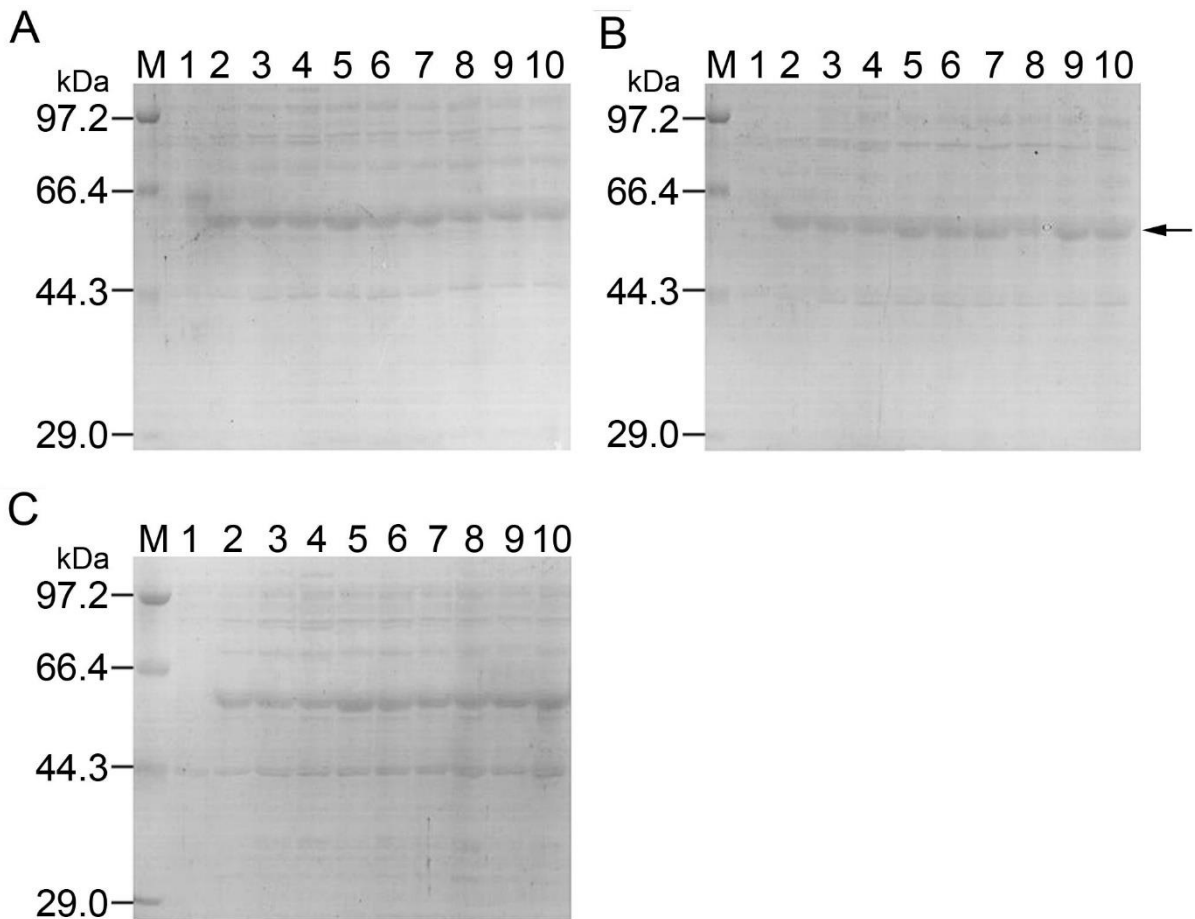


Figure 5. The orthogonal design experiment of the expression strain of flagellin protein C (FlaC) induced by isopropyl- β -D-thiogalactoside (IPTG). A, B, and C represent three repeated experiments. M, protein marker; 1, no induction strain. The 2, 3, and 4 mean a strain OD_{600} value of 0.5; IPTG final concentration of 0.1, 0.3, and 0.5 mmol/L; induction temperature of 28°C, 32°C, and 37°C; and induction time of 3, 8, and 12 hours. The 5, 6, and 7 mean a strain OD_{600} value of 0.8; IPTG final concentration of 0.1, 0.3, and 0.5 mmol/L; induction temperature of 32°C, 28°C, and 37°C; and induction time of 8, 12, and 3 hours. The 8, 9, and 10 mean a strain OD_{600} value of 1.0; final IPTG concentration of 0.1, 0.3, and 0.5 mmol/L; induction temperature of 32°C, 37°C, and 28°C; and induction time of 12, 3, and 8 hours.

5. Discussion

The FlaC protein has good immunogenicity, can stimulate an immune response, and has a potential application in a vaccine (10). In this study, the FlaC protein was obtained by molecular cloning and purification, and the mouse polyclonal antiserum was prepared with good specificity. The titre of FlaC protein antiserum reached 1:3200. Compared with monoclonal antibodies, polyclonal antibodies are more convenient and economical to prepare (13, 14) and are widely used in the immune protection function analysis of the protein (15). This study obtained the FlaC protein antiserum, which lays the foundation for the immunological function of FlaC protein.

Our research showed that there was interaction between FlaC protein antiserum and major aquaculture pathogenic *Vibrio* of *V. alginolyticus*

and *V. parahaemolyticus*; they formed antigen–antibody complexes, which probably provided for antigen presentation (16, 17). Therefore, the body's immune system can easily identify *Vibrio* with FlaC protein antiserum mediation and eliminate pathogenic bacteria. Thus, the FlaC protein was expected to display good immunogenicity. Some experiments have found that the FlaC protein provided immune protection for *L. sanguineus* against *V. alginolyticus* infection (10, 18). This study showed that the FlaC protein had a significant immune protection function (50%) against *V. alginolyticus* infection and some immune protection function (41.66%) against *V. parahaemolyticus*, and it laid a theoretical foundation for developing FlaC immunization agents.

Table 2. Induction expression conditions of FlaC strain

No.	A	B (mmol/L)	C (h)	D (°C)	Optical Density/SD ($\times 10^4$)
1	0.5	0.1	3	28	4.708 \pm 0.393
2	0.5	0.3	8	32	5.957 \pm 0.242
3	0.5	0.5	12	37	7.310 \pm 0.070
4	0.8	0.1	8	37	7.534 \pm 0.299
5	0.8	0.3	12	28	6.311 \pm 0.579
6	0.8	0.5	3	32	5.220 \pm 0.215
7	1.0	0.1	12	32	5.575 \pm 0.292
8	1.0	0.3	3	37	5.726 \pm 0.241
9	1.0	0.5	8	28	4.016 \pm 0.260
K1 (mean value 1)	5.992	5.999	5.518	6.012	
K2 (mean value 2)	6.355	5.998	6.527	5.584	
K3 (mean value 3)	5.107	5.515	6.399	5.859	
Range analysis	1.249	0.484	1.009	0.428	

A, B, C, and D mean the induction strain OD₆₀₀ value, induction of IPTG concentration, induction time, and induction temperature, respectively. Compared to the analysis size of the mean value of K1, K2, and K3, the optimal inducing expression condition of FlaC protein was A2, B1, C2, and D1, respectively.

In order to obtain the FlaC protein by large-scale fermentation, different factors and levels were set, using an L₉(3⁴) orthogonal design model (19). We found that the optimal induction conditions of FlaC protein were a strain OD₆₀₀ value of 0.8, final IPTG concentration of 0.1 mmol/L, an induction time of 8 hours, and an induction temperature of 28°C. IPTG is an active inducer of β -galactosidase, and a high concentration of IPTG has some cytotoxicity, inhibiting protein expression (20, 21); this fact is consistent with how our research showed that the low concentration of IPTG (0.1 mmol/L) improved FlaC protein expression. Some researchers have found that the protein expression was inhibited when induced for a long time (22, 23), and our research showed the same. A suitable induction time is favourable to the protein expression, and the cost of raw materials is saved compared to using a blind setting for induction time (24). To further verify this conclusion, our research showed that the 8-hour induction time was the best inducement condition. Thus, we suggest that the actual fermentation production would be best with strain induction at a logarithmic growth period, with a

low concentration of IPTG, at a suitable induction time, and at a low temperature.

Table 3. Variance results of FlaC protein expression

Factor	Optical Density Value Analysis		
	Mean Square	F-value	P-value*
A	3.718	37.191	0
B	0.624	6.237	0.009
C	3.139	31.392	0
D	8.027	80.284	0

A, B, C, and D mean strain OD₆₀₀ value, IPTG concentration, induction time, and induction temperature, respectively. *P < 0.05 means the factor has a significant difference from the control group.

6. Conclusions

The present study obtained the expression strain of *V. alginolyticus* FlaC protein, and the optimal fermentation conditions were determined. The FlaC protein was purified and antiserum was prepared, and it was confirmed that the FlaC protein possesses a preferable immunogenicity and excellent immune protection effect on *V. alginolyticus* and *V. parahaemolyticus* infection. Our findings would provide a scientific basis

for the vaccine development on major aquaculture pathogenic *Vibrio*.

Conflict of Interest

The authors declare no conflict of interest.

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