Fish and Shellfish Immunology 86 (2019) 143–151

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Identification of outer membrane protein TolC as the major adhesin and potential vaccine candidate for *Vibrio harveyi* in hybrid grouper, *Epinephelus fuscoguttatus* (♀) × *E. lanceolatus* (♂)

Zhiming Zhu[a,b,c], Chuanfu Dong[b,c,*], Shaoping Weng[b,c], Jianguo He[a,b,c,**]

[a] Guangdong Provincial Key Laboratory of Marine Resources and Coastal Engineering, School of Marine Sciences, Sun Yat-sen University, Guangzhou, 510275, PR China
[b] State Key Laboratory of Biocontrol / MOE Key Laboratory of Aquatic Product Safety, School of Life Sciences, Sun Yat-sen University, Guangzhou, 510275, PR China
[c] Institute of Aquatic Economic Animals, and Guangdong Province Key Laboratory for Aquatic Economic Animals, Sun Yat-sen University, Guangzhou, 510275, PR China

ARTICLE INFO

Keywords:
Vibrio harveyi
TolC
Major adhesin
Adhesion function
Immunoprotectivity

ABSTRACT

Vibrio harveyi is a serious pathogen of scale drop and muscle necrosis disease in marine commercial fishes. Adhesion to and colonization of the host cells surfaces is the first and crucial step for pathogenic bacterial infection, which is usually mediated by outer membrane proteins (Omps). The objectives of this study were to identify the major adhesin in Omps that plays the essential role in adhesion of *V. harveyi* to the host cells, and to assess the potential of this adhesin as a vaccine candidate for *V. harveyi* infection. We observed that pathogenic *V. harveyi* adhered to the surface of grouper embryonic cells (GEM cells) and induced apoptosis of them. Native Omps were extracted from nine different *V. harveyi* strains, and five common Omp bands were isolated by SDS-PAGE analysis. Western blot analysis and an anti-native Omp antibodies blocking assay indicated that one strong and several weak immunoreactivity Omps bands presence. Next, a total of five Omps, including TolC, Agg (Agglutination protein), Omp47, Fla (Flagellin), and OmpW, were identified and their encoding genes were cloned, characterized, and expressed in *E. coli*. The purified recombinant TolC could competitively inhibit the invasion of *V. harveyi* to GEM cells *in vitro*, and anti-TolC antibody also could significantly block the adhesion of *V. harveyi* to GEM cells. When used to immunize hybrid groupers, the recombinant TolC could confer significant protection to fish against experimental *V. harveyi* challenge. These data suggested that outer membrane protein TolC functions as a major adhesin in *V. harveyi* and could be a potential vaccine candidate for *V. harveyi* infection.

1. Introduction

V. harveyi is a marine Gram-negative bacterium that reported as a serious pathogen for a wide range of marine vertebrates and invertebrates worldwide [1]. The disease caused by *V. harveyi* is widely distributed in the coastal areas of China, which was shown to cause "scale drop and muscle necrosis" disease and acute mortality in *Epinephelus* spp., leading to huge economic losses in marine fish farming [2–4]. A variety of virulence factors, such as lipopolysaccharides, extracellular products (alkaline proteases, metalloprotease, phospholipase and hemolysins), and the quorum sensing system, were reported to be contributable to the overall virulence of the pathogenic *V. harveyi* [5–8]. However, the first and crucial step in host-pathogen interactions and tissue colonization is the adherence of pathogenic bacteria to the surface of the host cells [9,10]. Invasion following adhesion allows bacteria to evade the humoral immune response and exert toxic action on host tissues [11].

The pathogen adhesion to and colonization of host cells surfaces are usually mediated by adhesins such as outer membrane proteins (Omps), which play an essential role in causing infection. Omps represent a large group of β-barrel proteins found in the outer membrane of Gram-negative bacteria, which were reported as important active pathogenic components that mediate the initial adhesion of bacteria to host cells, modulate host-pathogen interactions, impact the overall survival of the organism and propagate virulence factors [11,12]. Furthermore, bacteria can secrete a wide range of substrates, such as small molecules and proteins that are toxic for the host, by several types of secretion systems, which are also primarily made up of Omps [9,13]. The adhesion function of Omps has been well documented in several fish pathogens such as *Aeromonas* spp. and *Vibrio* spp. [14–17]. In addition, bacterial

* Corresponding author. School of Marine Sciences, Sun Yat-sen University, No. 135, Xingangxi Road, Guangzhou, 510275, PR China.
** Corresponding author. School of Marine Sciences, Sun Yat-sen University, No. 135, Xingangxi Road, Guangzhou, 510275, PR China.
E-mail addresses: dongchfu@mail.sysu.edu.cn (C. Dong), lsshjg@mail.sysu.edu.cn (J. He).

https://doi.org/10.1016/j.fsi.2018.11.037
Received 20 July 2018; Received in revised form 13 November 2018; Accepted 15 November 2018
Available online 16 November 2018
1050-4648/

adhesion to host cells mediated by Omps can stimulate the host immune response because outer membrane components are easily recognized as foreign substances by immunological defense systems of the host to induce the upregulation of pro-inflammatory cytokines. These findings suggest that Omps play important roles both in the process of bacterial invasion and stimulation of the host immune response.

The traditional control measure of the disease caused by *V. harveyi* is antibiotic treatment. However, multiple antibiotic resistance occurs in *V. harveyi* owing to antibiotic misuse in the aquaculture industry, making the therapy difficult and resulting in a catastrophic risk for aquaculture [2,18,19]. Vaccination has been practiced for a long history and it is one of the most effective methods for controlling infectious diseases in humans and animals. Therefore, an important marine aquaculture priority is the development of an effective vaccine for *V. harveyi* infection. However, no commercial vaccine for protection against *V. harveyi* is available in China owing to the diversity of the surface antigens in *V. harveyi*. The common protective antigens of *V. harveyi* such as Omps would be potential candidates for a vaccine against this bacterium because they are not only involved in the process of adhesion and invasion to the host but also associated with immunogenicity [20–22]. Thus, it may be possible to prevent *V. harveyi* infection by blocking bacterial invasion into fish with antibodies against the adhesion of Omps. Although several Omps and maltoporin in *V. harveyi* have been reported that can provide some immunogenicity to protect fish against *Vibrio* spp. infection [20,23], information on the *V. harveyi* major adhesin and its immunogenicity are still not available.

In the present study, a total of five Omps with immunoreactivity were identified from pathogenic *V. harveyi*, and their encoding genes were cloned, characterized and expressed in *E. coli*. A 48.1 kDa outer membrane protein TolC was identified as the major adhesin by western blot analysis and an adhesion function assay *in vitro*, and it was found that recombinant TolC could provide protective immunity to hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *E. lanceolatus* ♂) against *V. harveyi*.

2. Materials and methods

2.1. Assay of V. harveyi adhesion to host cells

Pathogenic *V. harveyi* GDRP-M49 was obtained from diseased hybrid grouper, Guangdong province in China. The pathogen was routinely cultured in 2216 medium and/or on 2216 agar plate at 28 °C. *V. harveyi* cell pellets were collected by centrifugation, washed three times and adjusted to 10^9 CFU/ml with sterilized PBS buffer. Bacterial suspension of "*V. harveyi*-DMEM" was prepared with *V. harveyi* suspension as described above and with DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum (FBS) in the proportion of 1:100.

Grouper embryonic cells (GEM cells) were cultured in ambient air with 5% CO_2 at 27 °C in DMEM containing 10% FBS for 24 h and then were transferred to and cultured in 24-well culture plates for 24–48 h to obtain monolayer cells. The monolayer cells were washed three times with sterilized PBS buffer, and then 500 μl "*V. harveyi*-DMEM" suspension was added to each well for culture under the same conditions. The cell morphology was observed under an inverted microscope at 15-min intervals within 2 h. For each time-point, four wells of the 24-well culture plate were randomly selected and washed three times with PBS to remove un-adhered *V. harveyi* pellets. To each well, 250 μl trypsin enzyme was added to digest the GEM cells, and then 250 μl DMEM containing 10% FBS was added to neutralize the activity of the trypsin enzyme. The digested cells in each well were collected, and the number of *V. harveyi* were counted by the conventional plate count method.

To investigate the apoptosis of GEM cells induced by *V. harveyi*, the cell apoptosis was detected by flow cytometry (BD Accuri™ C6, BD company, USA) at the 0, 30, 60 min and 2 h post incubation with "*V. harveyi*–DMEM" suspension as described above using the Annexin V-FITC/PI Apoptosis Detection Kit (ThermoFisher Scientific) according to the operation manual.

2.2. V. harveyi Omps extraction and western blot analysis

A total of nine *V. harveyi* strains that were identified from diseased fish by our laboratory were used for native Omps (n-Omps) extraction [2], which were grown in 2216 broth at 28 °C for 12–16 h. The *V. harveyi* cells were collected by centrifugation at 6000*g* for 10 min at 4 °C. After washing three times with PBS, the Omps were extracted according to the method as previously described [15].

The composition and molecular mass of the n-Omps were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Anti-*V. harveyi* antibody was generated by New Zealand rabbit immunization. Briefly, the rabbit was injected with *V. harveyi* GDRP-M49 cells that were inactivated by formalin (FKC) in PBS and emulsified with an equal volume of Freund's complete adjuvant. One week after the first injection, the rabbit was injected once a week for two consecutive weeks with FKC emulsified with an equal volume of Freund's incomplete adjuvant, and then boostered without adjuvant at the fourth week. The serum was collected from the side veins of the rabbit's ear by centrifuged at 3000 rpm, 4 °C, and the antibody titer was detected by ELISA.

Next, the immune-reactivity of n-Omps was examined by western blot analysis, performed by using the anti-*V. harveyi* antibody as the first antibody and HRP-labelling sheep anti-rabbit IgG antibody as the second antibody.

2.3. Anti-native Omps antibodies blocking assay

Anti-native Omps (anti-*n*-Omps) antibodies were also generated by New Zealand rabbit immunization. The n-Omps that were extracted from the *V. harveyi* strain were isolated by SDS-PAGE, and then each band of Omp was cut off from the gel, and ground in PBS for antibodies preparation as described above.

A bacterial suspension of "*V. harveyi*-DMEM" and anti-*n*-Omps antibody was mixed completely in the proportion of 100:1, incubated for 30 min and added to coverslip cultures of GEM cells at 500 μl for each well of a 24-well plate. The infected monolayers were incubated for 90 min under the same conditions. No adherent bacteria were removed from the monolayers by washing three times with sterilized PBS. The cells were fixed with methanol: acetic acid (3:1) for 15 min and stained with Giemsa 10% by the conventional method. The coverslips were mounted on glass slides and viewed by light microscopy. For quantitative analysis, the infected monolayer cells that were cultured in 24-well plates were collected and *V. harveyi* was counted as described above.

2.4. Omps identification, coding genes cloning and analysis

The common Omps bands that exhibited immunoreactivity with anti-*V. harveyi* antibody in these nine *V. harveyi* strains were cut carefully from the polyacrylamide gel, and then identified by Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS) (Guangzhou Fitgene Biotechnology Co., Ltd, China). Homologous genes of these identified Omps in *V. harveyi* were obtained from BLAST search on line Uniprot database (http://www.uniprot.org/).

The specific primers of the Omps encoding genes were designed and synthesized according to the nucleotide sequences of homologous genes from the Uniprot database. In addition, the protective bases **CGC** and **CCG** and the restriction sites for *Bam*H I (*GGATCC*) and *Xho*I (*CTCGAG*) were added to the 5'-terminal ends of the forward and reverse primers, respectively. The sequences of specific primers that for Omps encoding genes amplification were showed as supplementary data. Genomic DNA of *V. harveyi* GDRP-M49 was extracted with the TaKaRa MiniBEST

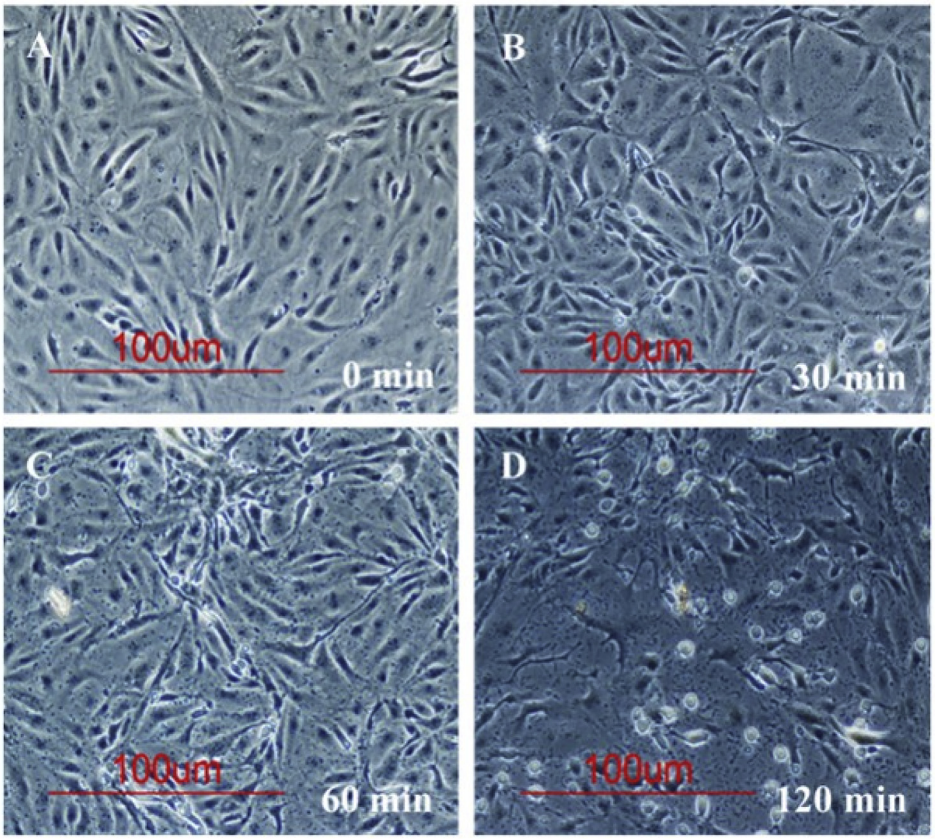

Fig. 1. *V. harveyi* induced morphological changes and apoptosis of GEM cells. The structure of normal GEM cells was integral and clear (A), *V. harveyi*-induced gross morphological changes (B and C), and apoptosis (D).

Bacteria Genomic DNA Extraction Kit Ver.3.0 (TaKaRa, Japan). PCR amplification was performed in a 50 μl volume containing 25 μl of Premix Taq (TaKaRa Taq Version 2.0 plus dye), 22 μl of ddH_2O, 1 μl of each primer (20 mM), and 1 μl of bacterial genome DNA (100 ng). PCR products were purified, ligated with the pMD-19T vector and transformed into competent *E. coli* DH 5α cells. Positive clones were sequenced and analyzed by the BLAST program of the National Center for Biotechnology Information (NCBI: https://blast.ncbi.nlm.nih.gov/Blast.cgi) for homology. A phylogenetic tree was constructed using the neighbor-joining algorithm of the MEGA 6.0 software with 1000 bootstrap replicates.

2.5. Construction of the expression system and the expression of Omps encoding genes in E. coli

The plasmid pET-32a (+) was used as the expression vector in *E. coli*, and *E. coli* BL21 (DE3) was used as the host for expression of the cloned genes in this study. Plasmids were extracted and digested by restriction enzymes, ligated with the pET-32a (+) vector, and transformed into competent *E. coli* BL21 (DE3). Positive clones were screened by plating on LB agar plates with 50 μg/ml kanamycin and confirmed by restriction enzyme digestion and DNA sequencing.

The positive clones were grown in LB broth with 50 μg/ml kanamycin at 37 °C. When OD_{600} reached 0.6, IPTG was added to a final concentration of 1 mM. Bacteria were harvested 6 h after the addition of IPTG. The expression of recombinant outer membrane proteins (r-Omps) were confirmed by SDS-PAGE, and then were purified by Ni^{2+} affinity chromatography using the ÄKTAprime FPLC Protein Purification System (GE Healthcare, Sweden). Next, anti-r-Omp antibodies were prepared by New Zealand rabbit immunization using purified r-Omp, respectively. To confirmed r-Omps have the same immunological properties as n-Omps, immuno-response activities of r-Omps were detected by western blot analysis using anti-*n*-Omps antibodies. In addition, immune cross-reactions of anti-r-Omps antibodies with n-Omps that were extracted from nine different strains of *V. harveyi* were also detected.

2.6. Adherence function assay of r-Omps

To detect the adherence function of r-Omps, two tests including a competitive inhibition assay and an anti-r-Omps antibodies blocking assay were conducted. The competitive inhibition assay was performed as described in previous with the following modifications [16]. First, 500 μl of the purified r-Omp (100 μg/ml) in PBS to be tested was added into individual respective wells, in which monolayer GEM cells had been prepared as described above, and then incubated for 45 min at 27 °C. In the control wells, an equal volume of BSA with same concentration was added. Four duplicates were performed for each sample in each experimental test. The inoculum was later removed and washed three times with sterilized PBS. Next, 500 μl of the "*V. harveyi*-DMEM" suspension was added to each well and incubated for 90 min under the same conditions. Then, the monolayer cells were washed three times with PBS to remove the bacteria that had not adhered to GEM cells, and then the cells were digested and adhered bacteria were counted as described above. The antibodies blocking assay was performed as described in section of 2.3 but the antibodies in the test were replaced with anti-r-Omps antibodies.

2.7. Fish protection studies

A total of 25 hybrid grouper *E. fuscoguttatus* ♀ × *E. lanceolatus* ♂ (weight, 18–20 g) were included in each experimental group of r-Omps,

which injected intramuscularly with 50 μg (0.1 ml) of r-Omp in PBS emulsified with a one-time mass of Montanide ISA 201 VG adjuvant (SEPPIC Shanghai, China). For the control, fish were injected with PBS emulsified with ISA 201 VG. The fish were kept in an indoor recirculation aquaculture system with water temperature of 24–26 °C and 26‰ salinity, and fed with commercial food pellets twice daily. Three weeks after the injection, fish were challenged with *V. harveyi* GDRP-M49 at 10^6 CFU per fish. The mortality of the fish was recorded within 14 days, and relative percent survival (RPS) was calculated as (1- (% immunized mortality/% control mortality)) × 100%.

2.8. Statistical analysis

Statistical analysis of the data was performed by one-way ANOVA and LSD multiple range analysis using SPSS 17.0. Results were presented as the means ± SD and differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. V. harveyi adhesion to and induction of host cell apoptosis

Before being challenged with *V. harveyi*, the structure of the GEM cells was integral and clear (Fig. 1 A). However, *V. harveyi* induced gross morphological changes of GEM cells, with some cells becoming atrophied after 30 min (Fig. 1 B), and a few cells dying after 60 min (Fig. 1C). Subsequently, a large number of GEM cells were ruptured, died and floated in the DMEM medium at 120 min post *V. harveyi* challenge (Fig. 1 D). Accordingly, the quantitative analysis showed that the number of *V. harveyi* that adhered to GEM cells rapidly increased with increasing incubation time, which reach a peak at the 90th min and then decreased, as shown in Fig. 2. This result is likely caused by the apoptosis of the GEM cells as shown in Fig. 1, because those *V. harveyi* cells adhered to floating dead GEM cells were washed out by PBS buffer during the quantitative analysis.

The analysis results of GEM cells apoptosis which induced by *V. harveyi* was showed in Fig. 3. Specifically, few apoptosis cells could be detected in the control group (Fig. 3 A) and the short-time (30 min) *V. harveyi*-infection group (Fig. 3 B), which the rates of early apoptotic cells and late apoptotic cells were 3.57% and 2.37% in the control group and were 4.21% and 3.37% in the short-time infection group, respectively. However, the number of apoptosis cells were increased rapidly with the increasing *V. harveyi*-infection time, which the rates of early apoptotic cells and late apoptotic cells were 7.83% and 3.95% at 60 min post *V. harveyi* infection (Fig. 3C), and then reached to 21.30% and 7.12% at 2 h post infection (Fig. 3 D). These results indicated that *V. harveyi* infection induced apoptosis of GEM cells, and the apoptotic rates depended on the infection time.

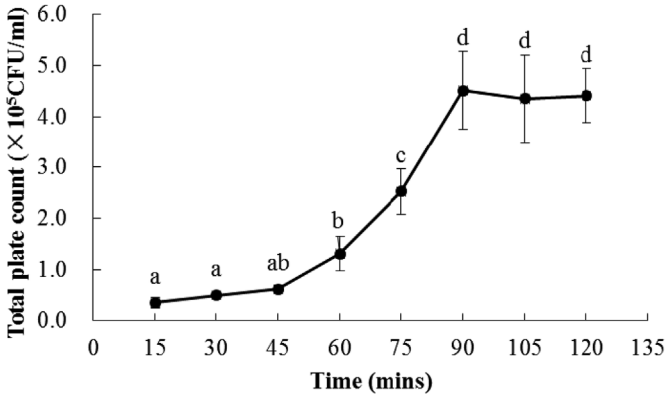

Fig. 2. The number of *V. harveyi* adhesion to GEM cells at different time intervals. Different letters above lanes represent significant differences among time-points, one-way ANOVA, $p < 0.05$.

3.2. SDS-PAGE and western blot analysis of n-Omps in V. harveyi

In the present study, n-Omps were extracted from nine different *V. harveyi* strains. Results of SDS-PAGE analysis showed that there were five abundant Omps bands observed (Fig. 4 A). In addition, the results of western blot analysis by anti-*V. harveyi* antibody showed that there was a strong immunoreactivity Omps band of approximately 40 kDa and several weak immunoreactivity Omps bands (Fig. 4 B). These experimental results indicated that different *V. harveyi* strains have several common immunoreactive Omps.

3.3. Anti-n-Omps antibodies blocking V. harveyi adhesion to host cells

There were a large number of *V. harveyi* adhesion to the surface of the GEM cells after incubation together (Fig. 5 A), and the negative serum collected from unimmunized New Zealand rabbit can minimally block *V. harveyi* adhesion to GEM cells (Fig. 5 B). However, the adhesion of *V. harveyi* to GEM cells was clearly prevented by anti-P1 antibody, resulting in markedly decreased *V. harveyi* adhesion numbers on GEM cell surfaces (Fig. 5C).

In addition, the quantitative analysis results showed that the five anti-*n*-Omps antibodies of *V. harveyi* have varying degrees of prevention of *V. harveyi* adhesion to GEM cells (Fig. 6). The number of *V. harveyi* adhesion to host cells were largely decreased by 33.33% in anti-P1 antibody group and by 28.09%, 27.03% and 25.27% in anti-P2, anti-P3 and anti-P5 antibodies groups, respectively. However, it was found that the adhesion numbers of *V. harveyi* did not decrease in the anti-P4 antibody group. These results indicated that the Omps within the protein bands P1, P2, P3 and P5 could play an important role in *V. harveyi* adhesion to host cells.

3.4. Identification, cloning and expression of Omps

Based on the results of western blot analysis, we identified five n-Omps from the P1, P2, P3 and P5 protein bands of *V. harveyi* by the MALDI-TOF-MS method, which were identified as TolC (48.1 kDa), Agg (Agglutination protein, 48.9 kDa), Omp47 (Omp of 47 kDa, 45.4 kDa), Fla (flagellin, 40.2 kDa) and OmpW (23.2 kDa), respectively, in the Uniprot database (http://www.uniprot.org/) (Table 1).

The five n-Omps encoding genes were successfully amplification as shown in Fig. 7 (A). Gene sequencing and homology analysis indicated that the amplified n-Omps encoding genes share remarkable homology with standard strains of *V. harveyi* ATCC 33843 and ATCC 43516 (Fig. 7, B).

As shown in Fig. 8, the five n-Omps were successfully expressed and purified in the present study. The mass of r-Omps was approximately 66.0 kDa of r-TolC, 66.8 kDa of r-Agg, 63.3 kDa of r-Omp47, 58.1 kDa of r-Fla and 41.1 kDa of OmpW. The mass of r-Omps were larger than n-Omps because a His-tag of 17.93 kDa was contained at the N-terminal of the r-Omps. The r-Omps were purified by Ni^{2+} affinity chromatography. Western blot analysis suggested that these r-Omps can induce an immune response with anti-*n*-Omps antibodies, which confirmed that these r-Omps were successfully expressed. In addition, immune cross-reaction of anti-r-Omps antibodies with n-Omps were also observed, indicating the immunogenicity of r-Omps (Fig. 9).

3.5. Omps plays an important role in V. harveyi adhesion to host cells

The competitive-inhibition effect of the r-Omps was tested *in vitro* with GEM cells. The data showed that the r-Omps inhibited *V. harveyi* adhesion to GEM cells (Fig. 10). Briefly, the number of *V. harveyi* adhesion to GEM cells in the r-TolC group was reduced at 36.22% compared to the control group, which exhibited the greatest inhibitory effect on *V. harveyi* invasion into the GEM cells. However, the decrease of

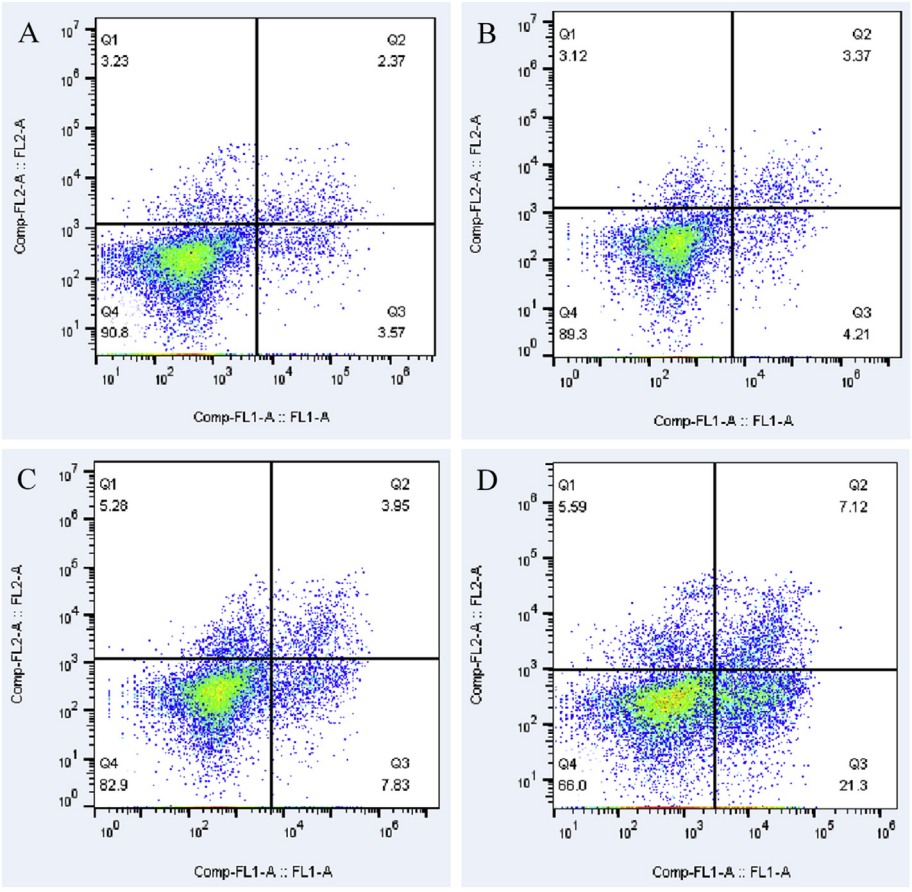

Fig. 3. The apoptosis of GEM cells induced by *V. harveyi*. Groups of A, B, C and D represent the *V. harveyi* infection time were 0, 30, 60 min and 2 h, respectively.

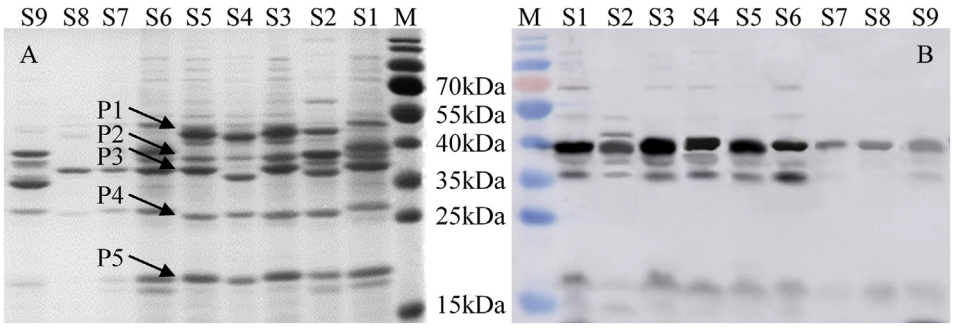

Fig. 4. SDS-PAGE (A) and western blot (B) analysis of n-Omps extracted in *V. harveyi*. M, marker; S1-S9, n-Omps extracted from the nine different strains of *V. harveyi*, respectively; P1-P5, common Omps in different *V. harveyi* strains.

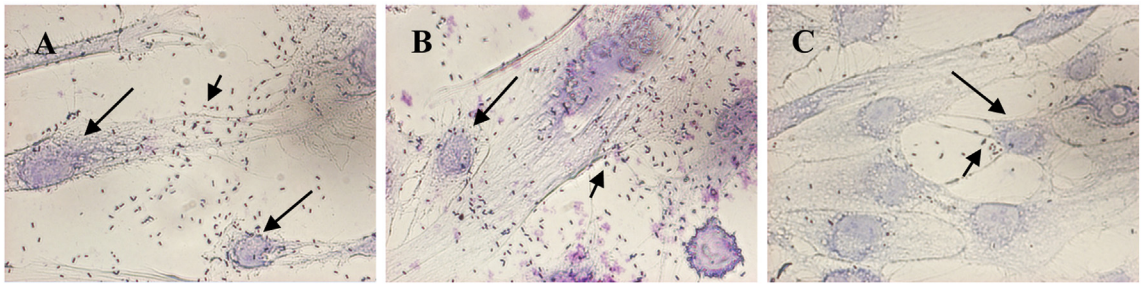

Fig. 5. Anti-P1 antibody blocked *V. harveyi* adhesion to host cells. A, *V. harveyi* adhesion to the surface of GEM cells; B, negative serum cannot block *V. harveyi* adhesion to GEM cells; C, anti-P1 antibody blocked the adhesion of *V. harveyi* to GEM cells. Black short and long arrows represent the *V. harveyi* and GEM cells, respectively.

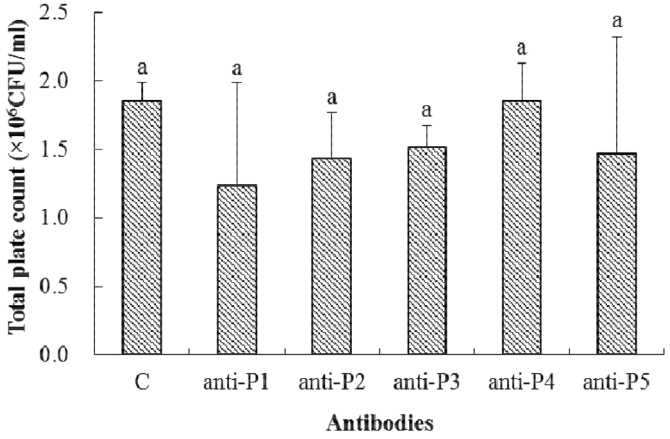

Fig. 6. Anti-*n*-Omps antibodies blocked *V. harveyi* adhesion to GEM cells. C, negative serum; anti-P1 to anti-P5, antibody serum of anti-*n*-Omps P1 to P5 of *V. harveyi*, respectively; the same letter above lanes represents no significant difference among groups, one-way ANOVA, $p \geq 0.05$.

Table 1
Identification of n-Omps in *V. harveyi* by MALDI-TOF-MS analysis.

Proteins	Accession	Score	Matches	Sequences	emPAI	Mass(Da)
Omp TolC	D0X7A6	380	11 (9)	10 (9)	0.82	48114
Agglutination protein (Agg)	D0XI94	1310	25 (21)	17 (13)	2.93	48896
Omp47	D0X5W8	159	6 (5)	3 (3)	0.42	45390
Flagellin (Fla)	D0X8J4	872	19 (18)	13 (12)	3.15	40207
OmpW	D0X5G7	573	24 (18)	3 (3)	0.96	23159

V. harveyi adhesion number in the r-Agg, r-Omp47, r-Fla and r-OmpW groups ranged from 13.21% to 20.39%. The results showed that TolC played the major role in *V. harveyi* adhesion to host cells, indicating it is probably the major adhesin of *V. harveyi*.

To further confirm the adhesion function of Omps and find the major adhesin of *V. harveyi*, we prepared five anti-r-Omp antibodies, including anti-r-TolC, anti-r-Agg, anti-r-Omp47, anti-r-Fla and anti-r-OmpW antibodies, to detect their blocking effect. The results showed that anti-r-TolC antibody can significantly ($p < 0.05$) reduce the number of *V. harveyi* adhesion to GEM cells at 37.68%, which was also markedly lower than that in other groups (Fig. 11). Therefore, we concluded that the TolC is the major adhesin of *V. harveyi* and could be a highly conserved component in different *V. harveyi* strains.

3.6. Protection of r-Omps in hybrid groupers

To test the effectiveness of the r-Omps as a vaccine, hybrid groupers were immunized with the r-Omps and then challenged with virulent *V. harveyi*, and the results were showed in Fig. 12. The mortality of un-immunized fish in the control group (injected with PBS) was 76.00%. The lowest mortality of fish was observed in the r-TolC group at 20.00%, whose relative percent survival (RPS) reached to 73.68%. However, the RPS of the r-Agg, r-Omp47, r-Fla and r-OmpW groups were 30.75%, 5.26%, 21.05% and 15.79%, respectively. Thus, the r-TolC exhibited the highest RPS among r-Omp immunized groups, indicating that the TolC has potential as a subunit vaccine candidate for *V. harveyi* infection.

4. Discussions

Bacteria often attach to and live in close association with the host surface by specific proteinaceous adhesins that enable them to resist physical removal such as hydrodynamic shear-forces. Importantly, adhesion to and colonization of the host is a critical step in the pathogenesis of many bacterial pathogen infections [9]. Sometimes, the adhesive factor can directly contribute to the virulence of bacterial pathogens [24]. In the present study, *V. harveyi* can also adhere to the GEM cells surfaces, suggesting that there are specific adhesins existing on the surface of *V. harveyi* cells. However, the adhesion number of *V. harveyi* peaked at the 90th min and declined thereafter, and many GEM cells experienced atrophy, rupture and apoptosis, which resulted from the specific interaction of the invading *V. harveyi* and host cells. Specifically, an invading bacterium depends on the interaction between host defenses and microbial virulence factors such as the ability to produce toxins, to resist the immune defenses of the host or disrupt host-cell barrier function and tight junctions, and then to target and colonize the cell surfaces and/or induce apoptosis [9,13,25]. The result is that those toxins secreted by, and the downstream processes initiated by *V. harveyi* induced characteristic morphological changes and apoptosis of the host cells in the present study.

Many bacterial pathogens can express an array of different adhesins that enable them to attach to attachment sites such as proteins, glycoproteins and glycolipids existing on the host-cell surfaces [9,10]. Omps, which have been characterized as extremely stable β-barrels structures, are almost exclusively found in the outer membrane of Gram-negative bacteria [13,26]. Their receptor-like domain or subunit that are exposed on the bacterial cell surfaces enable them to easily recognize and closely bind to the ligand located on the surfaces of the host cells. Therefore, Omps have been identified to be the most important adhesin in Gram-negative bacteria [14,27,28]. However, there was very limited information about the major adhesin in Omps that plays the most important role in *V. harveyi* adhesion to the host. In the present study, n-Omps extracted form *V. harveyi* showed strong immunoreactivity, and results of microscopy and quantitative analysis by bacterial counts also showed that the anti-*n*-Omps antibody can markedly inhibit the adhesion of *V. harveyi* to host cells. These results suggested that the Omps mediated the adhesion process of *V. harveyi* to host cells, and the attachment site of Omps, located on the outer membrane surface, can be blocked by the specific anti-*n*-Omps antibody. In the five r-Omps prepared by the prokaryotic expression system in the present study, the adhesion of *V. harveyi* to GEM cells was notably inhibited by r-TolC. In particular, the number of *V. harveyi* adhesion to GEM cells was also significantly decreased after pretreatment by anti-r-TolC antibody. Therefore, it was concluded that the TolC but not others Omps played

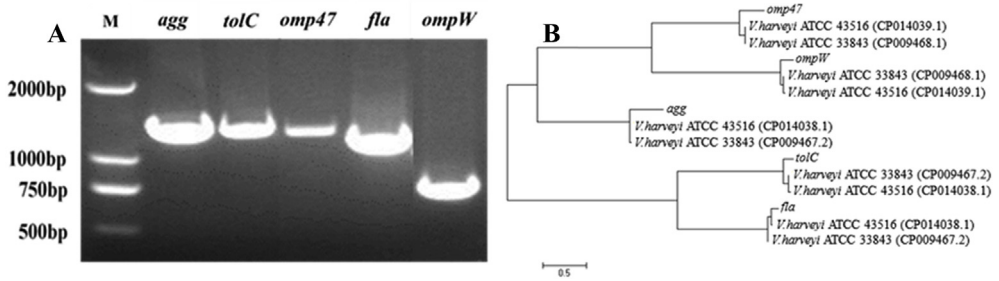

Fig. 7. PCR amplification (A) and homology analysis (B) of n-Omps encoding genes.

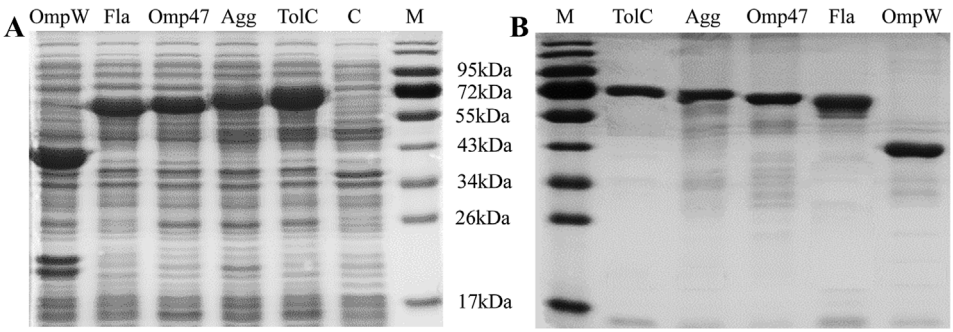

Fig. 8. Prokaryotic expression of n-Omps encoding genes (A) and purification of r-Omps (B).

the most important role in the process of *V. harveyi* adhesion to host cells.

Since bacterial pathogens adhere to host cells by adhesins, disruption of bacterial attachment by specific anti-adhesin antibodies seems to be an obvious way to incapacitate a potential pathogen. In fact, acquired immunity is largely directed against bacterial adhesins owing to their exposed epitopes on the cell surface, which suggests that bacterial adhesins are often excellent immunogens and have made bacterial adhesins prime targets for vaccine development. Thus, a great number of studies have reported the immunoprotection of Omps in fish bacterial pathogens. For example, a 43 kDa Omp was identified to be major adhesin of *Aeromonas hydrophila* and was indicated to have good immune protection [14,29], and the OmpA of *Edwardsiella tarda* was reported as a potential vaccine candidate for fish [30]. Omps, which are conserved across serotypes and highly immunogenic, have been used as good candidates in vaccine development for combating disease problems in commercial aquaculture.

Many types of Omps in *Vibrio* spp. have been also reported as effective vaccine candidates. For example, the OmpK, which was a shared antigen among *Vibrio* species, can induce the production of specific antibodies against OmpK and shows high efficacy of protection in immunized fish against *Vibrios* infection [31–33]. OmpU and LamB (maltoporin) were also reported as important antigens of *Vibrio* spp., which were more appropriate candidates as epitope-based vaccines against *Vibrio* species infection in aquatic animals [23,34]. In the present study, the process of *V. harveyi* adhesion to host cells was inhibited by r-TolC and anti-r-TolC antibody can also notably prevent *V. harveyi* attachment to host cells, indicating that it is possible to prevent *V. harveyi* infection by vaccination with TolC in fish. Indeed, an oil emulsion adjuvant vaccine prepared with r-TolC as the antigen showed good immuno-protective effect against *V. harveyi* infection in hybrid grouper, *E. fuscoguttatus* (♀) × *E. lanceolatus* (♂). The RPS of TolC in immunized fish reached 73.68%, which was a good protective rate that was higher than the other Omps identified in the present study. Moreover, the RPS was also higher than that of almost all of the 13 Omps of *V. harveyi* reported in a previous study [20]. Accordingly, the TolC is likely to be the major adhesin of *V. harveyi*, which would be a potential candidate of vaccine development against this bacterial infection in marine aquaculture.

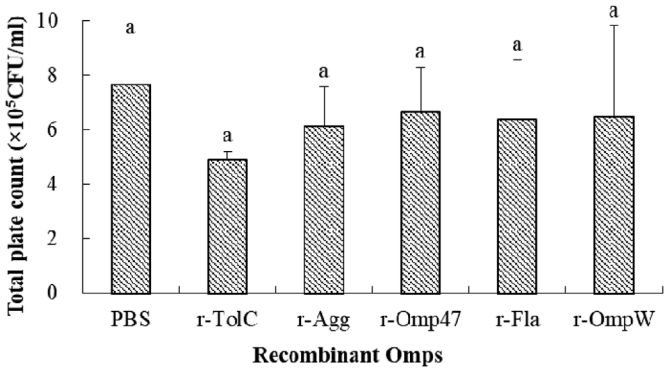

Fig. 10. Competitive inhibitory effect of r-Omps on the adhesion of *V. harveyi* to GEM cells. PBS, control group of no r-Omps but PBS added; r-TolC, r-Agg, r-Omp47, r-Fla and r-OmpW, 100 μg of r-Omp added, respectively; the same letter above lanes represents no significant difference among groups, one-way ANOVA, $p \geq 0.05$.

TolC is the outer membrane protein involved in the type I secretion system, and its crystal structure showed it is a trimeric 12-stranded α/β barrel comprising an α-helical trans-periplasmic tunnel embedded in the outer membrane by a contiguous β-barrel channel [35,36]. This structure establishes a specific tunnel across the outer membrane of Gram-negative bacteria. Therefore, TolC plays the most important role in the expulsion of diverse molecules, and previous studies also focused on its roles in antibacterial drugs efflux and proteins export from the cells [35–39]. Although TolC was also reported to be involved in the invasiveness of *Salmonella enterica* and required for colonization of *Vibrio cholera* [37,38,40], information on its adhesion function and immunogenicity is limited. In the present study, TolC is confirmed to not only mediate *V. harveyi* adhesion to host cells *in vitro*, but also provide effective protection against challenge with this virulent bacterium. These results provide a more comprehensive basis for further research of bacteria-host interactions and pathogenesis.

Acknowledgements

We are particularly grateful to the anonymous reviewers for

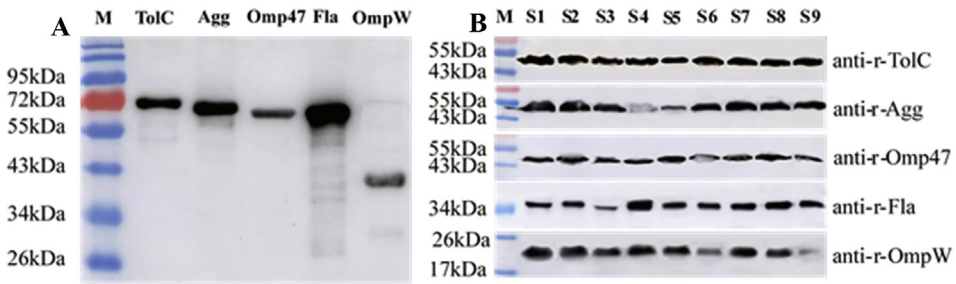

Fig. 9. Detection of anti-r-Omps antibodies (A) and cross immune reaction between anti-r-Omp antibodies and n-Omps (B). M, marker; the lanes of TolC, Agg, Omp47, Fla, OmpW represent the immune reaction bands of r-Omps with anti-*n*-Omps antibodies; S1-S9 represent the immune reaction bands of n-Omps extracted from nine different *V. harveyi* strains with anti-r-Omps antibodies.

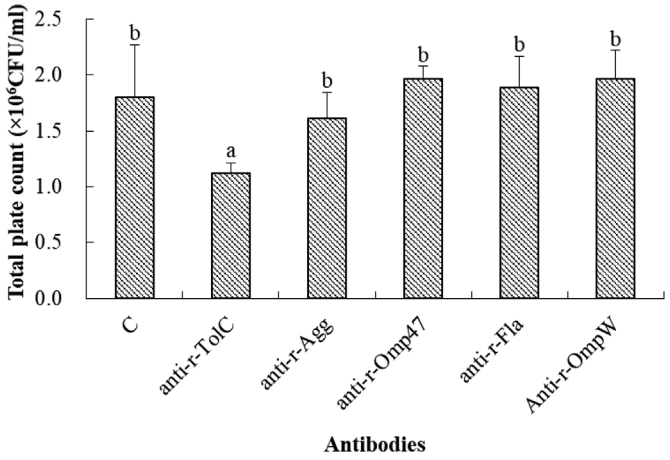

Fig. 11. Anti-r-Omps antibodies blocked the adhesion of *V. harveyi* to GEM cells. C, control group of negative serum; anti-r-TolC, anti-r-Agg, anti-r-Omp47, anti-r-Fla and anti-r-OmpW represent the anti-r-Omp antibodies groups, respectively. Different letters above lanes represent significant difference among groups, one-way ANOVA, $p < 0.05$.

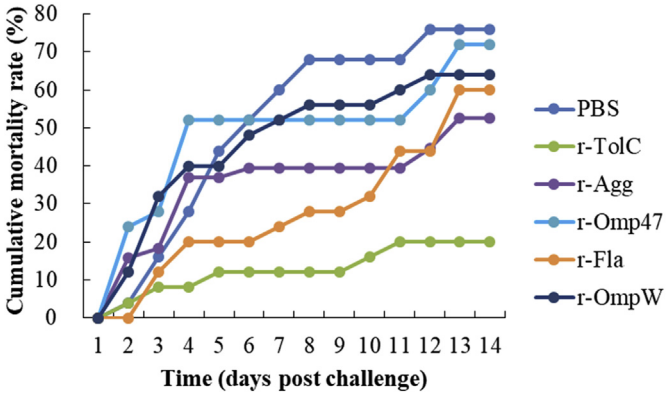

Fig. 12. Cumulative mortality of r-Omps-vaccinated hybrid groupers post virulent *V. harveyi* challenge.

providing the critical comments. This work was supported by the National Natural Science Foundation of China (Grant Number 31702380), China-ASEAN Center for Joint Research and Promotion of Marine Aquaculture Technology, and Fundamental Research Funds for the Central Universities (Grant Number 18lgpy30).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.11.037.

References

[1] B. Austin, X.H. Zhang, *Vibrio harveyi*: a significant pathogen of marine vertebrates and invertebrates, Lett. Appl. Microbiol. 43 (2) (2006) 119–124.
[2] Z.M. Zhu, C.F. Dong, S.P. Weng, J.G. He, The high prevalence of pathogenic *Vibrio harveyi* with multiple antibiotic resistance in scale drop and muscle necrosis disease of the hybrid grouper, *Epinephelus fuscoguttatus* (♀) × *E. lanceolatus* (♂), in China, J. Fish. Dis. 41 (4) (2018) 589–601.
[3] G.M. Shen, C.Y. Shi, C. Fan, D. Jia, S.Q. Wang, G.S. Xie, G.Y. Li, Z.L. Mo, J. Huang, Isolation, identification and pathogenicity of *Vibrio harveyi*, the causal agent of skin ulcer disease in juvenile hybrid groupers *Epinephelus fuscoguttatus* ♀ × *Epinephelus lanceolatus* ♂, J. Fish. Dis. 40 (10) (2017) 1351–1362.
[4] X.D. Xu, K.F. Liu, S.F. Wang, W.L. Guo, Z.Y. Xie, Y.C. Zhou, Identification of pathogenicity, investigation of virulent gene distribution and development of a virulent strain-specific detection PCR method for *Vibrio harveyi* isolated from Hainan Province and Guangdong Province, China, Aquaculture 468 (2017) 226–234.
[5] T. Defoirdt, R. Crab, T.K. Wood, P. Sorgeloos, W. Verstraete, P. Bossier, Quorum sensing-disrupting brominated furanones protect the gnotobiotic brine shrimp *Artemia franciscana* from pathogenic *Vibrio harveyi, Vibrio campbellii*, and *Vibrio parahaemolyticus* isolates, Appl. Environ. Microbiol. 72 (9) (2006) 6419–6423.
[6] P.C. Liu, K.K. Lee, S.N. Chen, Pathogenicity of different isolates of *Vibrio harveyi* in tiger prawn, *Penaeus monodon*, Lett. Appl. Microbiol. 22 (6) (1996) 413–416.
[7] F.M.I. Natrah, H.A. Darshanee Ruwandeepika, S. Pawar, I. Karunasagar, P. Sorgeloos, P. Bossier, T. Defoirdt, Regulation of virulence factors by quorum sensing in *Vibrio harveyi*, Vet. Microbiol. 154 (2011) 124–129.
[8] I. Zorrilla, S. Arijo, M. Chabrillon, P. Diaz, E. Martinez-Manzanares, M.C. Balebona, M.A. Morinigo, *Vibrio* species isolated from diseased farmed sole, *Solea senegalensis* (Kaup), and evaluation of the potential virulence role of their extracellular products, J. Fish. Dis. 26 (2003) 103–108.
[9] P. Klemm, M.A. Schembri, Bacterial adhesins: function and structure, Int. J. Med. Microbiol. 290 (1) (2000) 27–35.
[10] H.H. Niemann, W.-D. Schubert, D.W. Heinz, Adhesins and invasins of pathogenic bacteria: a structural view, Microb. Infect. 6 (1) (2004) 101–112.
[11] K.A. Kline, S. Falker, S. Dahlberg, S. Normark, B. Henriques-Normark, Bacterial adhesins in host-microbe interactions, Cell Host Microbe 5 (6) (2009) 580–592.
[12] D.E. Otzen, K.K. Andersen, Folding of outer membrane proteins, Arch. Biochem. Biophys. 531 (1–2) (2013) 34–43.
[13] A. Pavlova, H. Hwang, K. Lundquist, C. Balusek, J.C. Gumbart, Living on the edge: simulations of bacterial outer-membrane proteins, Biochim. Biophys. Acta 1858 (7) (2016) 1753–1759.
[14] H.M. Fang, R. Ge, Y.M. Sin, Cloning, characterisation and expression of *Aeromonas hydrophila* major adhesin, Fish Shellfish Immunol. 16 (5) (2004) 645–658.
[15] C.M. Rocha-De-Souza, A.V. Colombo, R. Hirata, A.L. Mattos-Guaraldi, L.H. Monteiro-Leal, J.O. Previato, A.C. Freitas, A.F.B. Andrade, Identification of a 43-kDa outer-membrane protein as an adhesin in *Aeromonas caviae*, J. Med. Microbiol. 50 (4) (2001) 313–319.
[16] H.M. Fang, K.C. Ling, Y.L. Tan, R. Ge, Y.M. Sin, *In vitro* inhibition of epithelial cell invasion by *Aeromonas hydrophila* and Vibrio species by fish *Aeromonas hydrophila* major adhesin, J. Fish. Dis. 21 (4) (1998) 273–280.
[17] X.Q. Liu, H.H. Gao, N. Xiao, Y. Liu, J.N. Li, L. Li, Outer membrane protein U (OmpU) mediates adhesion of *Vibrio mimicus* to host cells via two novel N-terminal motifs, PloS One 10 (3) (2015) e0119026.
[18] N. Stalin, P. Srinivasan, Molecular characterization of antibiotic resistant *Vibrio harveyi* isolated from shrimp aquaculture environment in the south east coast of India, Microb. Pathog. 97 (2016) 110–118.
[19] M. Raissy, M. Moumeni, M. Ansari, E. Rahimi, Antibiotic resistance pattern of some *Vibrio* strains isolated from seafood, Iran. J. Fish. Sci. 11 (3) (2012) 618–626.
[20] L.P. Yu, Y.H. Hu, B.G. Sun, L. Sun, Immunological study of the outer membrane proteins of *Vibrio harveyi*: insights that link immunoprotectivity to interference with bacterial infection, Fish Shellfish Immunol. 35 (4) (2013) 1293–1300.
[21] R.G. Gerlach, M. Hensel, Protein secretion systems and adhesins: the molecular armory of Gram-negative pathogens, Int. J. Med. Microbiol. 297 (6) (2007) 401–415.
[22] R.M. Khushiramani, B. Maiti, M. Shekar, S.K. Girisha, N. Akash, A. Deepanjali, I. Karunasagar, I. Karunasagar, Recombinant *Aeromonas hydrophila* outer membrane protein 48 (Omp48) induces a protective immune response against *Aeromonas hydrophila* and *Edwardsiella tarda*, Res. Microbiol. 163 (4) (2012) 286–291.
[23] J.S. Lun, C.Y. Xia, C.F. Yuan, Y.L. Zhang, M.Q. Zhong, T.W. Huang, Z. Hu, The outer membrane protein, LamB (maltoporin), is a versatile vaccine candidate among the *Vibrio* species, Vaccine 32 (7) (2014) 809–815.
[24] M. Liu, S. Chen, A novel adhesive factor contributing to the virulence of *Vibrio parahaemolyticus*, Sci. Rep. 5 (2015) 14449.
[25] T. Lynch, S. Livingstone, E. Buenaventura, E. Lutter, J. Fedwick, A.G. Buret, D. Graham, R. DeVinney, *Vibrio parahaemolyticus* disruption of epithelial cell tight junctions occurs independently of toxin production, Infect. Immun. 73 (3) (2005) 1275–1283.
[26] C.P. Moon, N.R. Zaccai, P.J. Fleming, D. Gessmann, K.G. Fleming, Membrane protein thermodynamic stability may serve as the energy sink for sorting in the periplasm, Proc. Natl. Acad. Sci. Unit. States Am. 110 (11) (2013) 4285–4290.
[27] S. Shin, G. Lu, M. Cai, K.S. Kim, *Escherichia coli* outer membrane protein A adheres to human brain microvascular endothelial cells, Biochem. Biophys. Res. Commun. 330 (4) (2005) 1199–1204.
[28] M. Muhlenkamp, P. Oberhettinger, J.C. Leo, D. Linke, M.S. Schutz, *Yersinia* adhesin A (YadA)–beauty & beast, Int. J. Med. Microbiol. 305 (2) (2015) 252–258.
[29] H.M. Fang, K.C. Ling, R. Ge, Y.M. Sin, Enhancement of protective immunity in blue gourami, *Trichogaster trichopterus* (Pallas), against *Aeromonas hydrophila* and *Vibrio anguillarum* by *A. hydrophila* major adhesin, J. Fish. Dis. 23 (2) (2000) 137–145.
[30] B. Maiti, M. Shetty, M. Shekar, I. Karunasagar, I. Karunasagar, Recombinant outer membrane protein A (OmpA) of *Edwardsiella tarda*, a potential vaccine candidate for fish, common carp, Microbiol. Res. 167 (1) (2011) 1–7.
[31] N.Q. Li, Z.H. Yang, J.J. Bai, X.Z. Fu, L.H. Liu, C.B. Shi, S.Q. Wu, A shared antigen among *Vibrio* species: outer membrane protein-OmpK as a versatile *Vibriosis* vaccine candidate in Orange-spotted grouper (*Epinephelus coioides*), Fish Shellfish Immunol. 28 (5–6) (2010) 952–956.
[32] H. Mohammed Abdullatif, N. Muliya Sankappa, S. Yassamin Nahil, K. Iddya, K. Indrani, Outer membrane protein K as a subunit vaccine against *V. anguillarum*, Aquaculture 354–355 (2012) 107–110.
[33] N.Q. Li, J.J. Bai, S.Q. Wu, X.Z. Fu, H.H. Lao, X. Ye, C.B. Shi, An outer membrane protein, OmpK, is an effective vaccine candidate for *Vibrio harveyi* in Orange-spotted grouper (*Epinephelus coioides*), Fish Shellfish Immunol. 25 (6) (2008) 829–833.
[34] J. Cen, X. Liu, J. Li, M. Zhang, W. Wang, Identification and immunogenicity of immunodominant mimotopes of outer membrane protein U (OmpU) of *Vibrio mimicus* from phage display peptide library, Fish Shellfish Immunol. 34 (1) (2013)

291–295.
[35] V. Koronakis, J. Eswaran, C. Hughes, Structure and function of TolC: the bacterial exit duct for proteins and drugs, Annu. Rev. Biochem. 73 (1) (2004) 467–489.
[36] V. Koronakis, A. Sharff, E. Koronakis, B. Luisi, C. Hughes, Crystal structure of the bacterial membrance protein TolC central to multidrug efflux and protein export, Nature 405 (22) (2000) 914–919.
[37] J.E. Bina, J.J. Mekalanos, *Vibrio cholerae* tolC is required for bile resistance and colonization, Infect. Immun. 69 (7) (2001) 4681–4685.
[38] A.M. Buckley, M.A. Webber, S. Cooles, L.P. Randall, R.M. La Ragione, M.J. Woodward, L.J. Piddock, The AcrAB-TolC efflux system of *Salmonella enterica* serovar Typhimurium plays a role in pathogenesis, Cell. Microbiol. 8 (5) (2006) 847–856.
[39] S. Baucheron, C. Mouline, K. Praud, E. Chaslus-Dancla, A. Cloeckaert, TolC but not AcrB is essential for multidrug-resistant *Salmonella enterica* serotype Typhimurium colonization of chicks, J. Antimicrob. Chemother. 55 (5) (2005) 707–712.
[40] I. Virlogeux-Payant, S. Baucheron, J. Pelet, J. Trotereau, E. Bottreau, P. Velge, A. Cloeckaert, TolC, but not AcrB, is involved in the invasiveness of multidrug-resistant *Salmonella enterica* serovar Typhimurium by increasing type III secretion system-1 expression, Int. J. Med. Microbiol. 298 (2008) 561–569.