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Immune response in *Lutjanus erythropterus* induced by the major outer membrane protein (OmpU) of *Vibrio alginolyticus*

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ABSTRACT: The outer membrane proteins (OMPs) of the marine aquatic animal pathogen *Vibrio alginolyticus* play an important role in the virulence of the bacterium and are potential candidates for vaccine development. In this study, the major 35.6 kDa OMP of *V. alginolyticus* was isolated by gel excision from the crude OMP fraction from *V. alginolyticus*. The sequence of the first 27 amino acid residues from the N-terminal end of the protein is ATV YKD GGT ELL VGG RVE FRG DFI GSD, which has high homology with OmpU proteins from other *Vibrio* spp. (92%). *Lutjanus erythropterus* were vaccinated with OmpU, and immunogenicity was confirmed by subsequent western blotting. Enzyme-linked immunosorbent assay (ELISA) analysis demonstrated that OmpU produced an observable antibody response in all sera of the vaccinated fish. *L. erythropterus* vaccinated with OmpU produced specific antibodies, and were highly resistant to infection with virulent *V. alginolyticus*. These results indicate that OmpU is an effective vaccine candidate against *V. alginolyticus* for *L. erythropterus*.

KEY WORDS: V*ibrio alginolyticus* · Outer membrane protein · OmpU · Immunogenicity · Vaccine

INTRODUCTION

Vibriosis is one of the most prevalent diseases in marine aquatic animals (Egidius 1987, de la Pena et al. 1993). Various *Vibrio* species have been identified as opportunistic pathogens that cause serious production losses in fish, shrimp and shellfish farms with mortality up to 100%, particularly in juvenile populations (Prayitno & Latchford 1995, Vandenberghe et al. 1998). *Vibrio alginolyticus* is one of the major *Vibrio* pathogens causing clinical signs of bacterial septicemia and skin ulcers in marine animals (Lee et al. 1996a, Zorrilla et al. 2003, Cai et al. 2007). Control of vibriosis through antibiotic treatment is not always successful and could be hazardous for the environment since resistant bacteria may be selected and the resistance may be transferred to aquatic animal or human pathogens. Therefore, the development of vaccines to protect aquatic animals against vibriosis is important (Kumar et al. 2007, Khushiramani et al. 2007, Li et al. 2008). Some virulence factors may contribute to the overall virulence of *V. alginolyticus*. These include extracellular products, endotoxins and adhesins (Lee et al. 1996b, Xu et al. 2005).

The outer membrane proteins (OMPs) of *Vibrio* are important adhesion factors. They are basically composed of protein, lipid and sugar, which could be easily recognized as foreign substances by the host's immunological defense systems (Qian et al. 2007). These OMPs play an important role in infection and pathogenicity in the host (Tsolis 2002). They possess high hydrophobicity, which is one of the important

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forces that macromolecules use to preserve their binding interactions. Therefore, OMPs can adhere to mucosal membranes, and antigen-presenting cells of gut-associated lymphoid tissues (GALT) can easily present OMPs as antigen to immune cells (Gordon & Alistair 1992). The OMPs of *Vibrio* are bile resistant and stimulate biofilm formation (Wang et al. 2003). Resistance to bile is important for bacteria to colonize the intestine of the fish host and biofilm formation provides an adaptive and survival advantage for bacteria in an aquatic environment (Olsson et al. 1996, 1998). The major OMP may be useful as a component of a *Vibrio* vaccine and also for diagnostic purposes (Jung et al. 2005, Hong et al. 2009).

The pathogenesis of *Vibrio alginolyticus* consists of gaining access to the host tissue, colonization and invasion. During colonization, surface materials of the organisms, such as lipopolysaccharide and OMPs, play an important role in the adhesion to host cells (Cheng et al. 2005, Hong et al. 2006). OMPs of *Vibrio* and their role have been mainly studied in *V. cholerae* (Sperandio et al. 1995), *V. anguillarum* (Waldbeser et al. 1993), *V. vulnificus* (Litwin & Byrne 1998) and *V. fischeri* (Aeckersberg et al. 2001), but there are very few reports on the immunological characterization of the major OMP from *V. alginolyticus*.

MATERIALS AND METHODS

Bacteria and culture conditions. *Vibrio alginolyticus* strain HY9901 was isolated from the diseased maricultured fish *Lutjanus erythropterus* at Zhanjiang, China. The isolate was preserved in Tryptone Soya Broth (TSB, Huangkai) supplemented with 2% NaCl and 20% glycerol at −80°C. The culture was retrieved on Tryptone Soya Agar (TSA, Huangkai) plates supplemented with 2% NaCl incubated at 28°C.

Crude OMP extraction. Isolation of OMPs was carried out using the method of Hamid & Jain (2008). The bacteria were harvested and washed 3 times with 10 mM Tris-HCl (pH 7.5), and 1.0 g (wet weight) of bacteria was extracted with 20 ml extraction buffer (10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 6 M urea) for 1 h at 4°C. The extract was dialyzed against distilled water for 3 d with frequent changes. The dialyzed material was centrifuged at 5400 × *g* for 1 h at 4°C, and the supernatant was collected and lyophilized with a lyophilizer (PowerDry PL3000 Freeze Dryer, Heto). The crude OMP extract was stored at −20°C until further use.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of crude OMPs. Protein samples were solubilized in Tris-HCl buffer supplemented with 2% sodium dodecyl sulfate (SDS) and 5% mercaptoethanol, and boiled at 100°C for 5 min. SDS-PAGE was carried out on a 12% separating gel with the discontinuous buffer system as described by Laemmli (1970). The gels were stained with 0.1% (wt/vol) Coomassie brilliant blue R-250 (Sigma) in 45.4% (vol/vol) methanol and 9.2% (vol/vol) glacial acetic acid, and destained with 5% (vol/vol) methanol and 7% (vol/vol) glacial acetic acid. The protein content of the crude OMPs was estimated using the method of Lowry et al. (1951).

Isolation and N-terminal amino acid sequence of 35.6 kDa OMP. The crude OMP preparation was fractionated on a preparative (3 mm thick, 12%) SDS-PAGE gel using the method of Hamid & Jain (2008). The 35.6 kDa OMP band was located by careful calculation of relative mobility values vis-à-vis the values for protein weight markers. The 35.6 kDa OMP band was excised from the gels, crushed finely, suspended in 2 volumes of elution buffer (0.025 M Tris-HCl, pH 8.3, 0.192 M glycine, 6 M urea), and incubated overnight at 4°C on a rocking platform. The supernatant was collected, and the gel pellet was re-extracted. The 2 supernatants were pooled, dialyzed at 4°C against distilled water to remove urea, and then lyophilized. The concentration of eluted protein was estimated using the method of Lowry et al. (1951), and its purity was assessed by SDS-PAGE and silver staining (Blum et al. 1987).

The 35.6 kDa protein (OmpU) was subjected to N-terminal sequence determination with an Applied Biosystems automated sequencer. This analysis was kindly performed by Shanghai GeneCore BioTechnologies, Shanghai, China.

Preparation of antiserum against *Vibrio alginolyticus* and the isolated OmpU. Antiserum against *V. alginolyticus* was raised in New Zealand white rabbits by injecting 2×10^5 colony forming units (CFU) of live bacteria in phosphate buffered saline (PBS, 10 mM, pH 7.2). All rabbits were boosted 4 wk later with the same antigen preparations. Four weeks after the booster injection, the rabbits were bled and the blood was clotted at room temperature for 1 h and then stored at 4°C overnight. The antiserum was used for western blot analysis.

Antiserum against the isolated OmpU was prepared according to the method of Khushiramani et al. (2007). This antiserum was used for analysis of antibody levels.

Western blot analysis. The crude OMPs from whole cells of *Vibrio alginolyticus* and the isolated 35.6 kDa OMP were electrophoresed by SDS-PAGE, using a 5% stacking gel and a 15% separating gel. The proteins were electrically transferred to nitrocellulose paper (0.45 mm pore size, Bio-Rad) using a semi-dry apparatus (Bio-Rad) as described by Towbin et al. (1979). The sera from rabbits vaccinated with *V. alginolyticus* were

used as primary antibody at a 1:100 dilution. The secondary antibody was peroxidase-conjugated goat-anti-rabbit IgG (Sigma) used at a 1:5000 dilution. The antibody-bound proteins were then visualized by 3,3′-diaminobenzidine (DAB).

Vaccine preparation and vaccination. The isolated OmpU (1 mg ml^{-1}) and sterile PBS (10 mM, pH 7.2) were each emulsified with an equal volume of Freund's incomplete adjuvant (Sigma). *Lutjanus erythropterus* weighing approximately 50 g were randomly divided into 2 groups with 70 fish in each group. Vaccination group was immunized by intramuscular injection with emulsified OmpU, and the control group with emulsified sterile PBS. After vaccination, fish were maintained at 25°C and fed commercial dry pellets daily.

Analysis of specific antibody levels. For 7 wk post-vaccination, 6 fish from each treatment group were assayed for antibody response against OmpU by enzyme-linked immunosorbent assay (ELISA) every week. The wells of 96-well plates were coated with the isolated OmpU (10 µg ml^{-1}). Fish serum samples (antibodies) of various dilutions were added to the microtiter plate, which had been blocked with 2% BSA. Antibody binding to the antigen was detected using rabbit antiserum against the isolated OmpU. Finally, plates were incubated with goat anti-rabbit IgG horseradish peroxidase (HRP) conjugate. The reaction was developed with the 3,3,5,5-tetramethylbenzidine (TMB) substrate in H_2O_2 and stopped with 2.0 M H_2SO_4. Optical density was measured at 450 nm using a microplate reader (µQuart, BioTek).

Bacterial challenge experiments. Virulent *Vibrio alginolyticus* strain HY9901 was cultured at 28°C in TSB medium for 18 h. On Day 50 post-immunization, 57 fish from the OmpU vaccination group were divided into 3 sub-groups. All sub-groups and the negative control group were then anaesthetized and challenged by intramuscular injection of 0.2 ml *V. alginolyticus* cell suspension (1×10^7 CFU ml^{-1}). Each sub-group of fish was then maintained in a separate tank with fresh running water at 25°C for 14 d. Dead fish were autopsied to determine the cause of death and to detect the presence of *V. alginolyticus* in the tissues by bacterial culture in thiosulfate citrate bile salts sucrose (TCBS) agar. Relative percentage survival (RPS) was calculated from the cumulative mortalities using:

$$\mathrm{RPS} = \left(\frac{1 - \text{Mortality of vaccinated fish}}{\text{Mortality of control fish}}\right) \times 100$$

Statistical analysis. Data are expressed as mean ± SD. Statistical analysis was performed to assess serum antibody levels and mortality for significance ($p < 0.05$) using Duncan's multiple range test approach. Probabilities of 0.05 or less were considered statistically significant.

RESULTS

Crude OMPs of *Vibrio alginolyticus*

The analysis of the crude OMPs by SDS-PAGE showed a complex electrophoretic profile having approximately 4 proteins with molecular masses between 29 and 97.4 kDa (Fig. 1A). The molecular masses of the 4 main proteins were calculated to be 47.8, 43.1, 39.3, and 35.6 kDa.

Isolation and N-terminal amino-terminal amino acid sequence of the 35.6 kDa OMP

The 35.6 kDa OMP is a major OMP of *Vibrio alginolyticus* with an apparent molecular mass of 35.6 kDa in SDS-PAGE (Fig. 1A, Lane 1), and it was isolated by excision from a preparative SDS-PAGE gel. The identity of the eluted material was confirmed by analytical SDS-PAGE, from which a single band of 35.6 kDa was obtained (Fig. 1A, Lane 2). From 56 mg crude OMPs loaded onto the preparative PAGE gel, 1.8 mg of the 35.6 kDa protein was obtained. The 35.6 kDa protein therefore comprises approximately 3.2% of the total crude OMPs of *V. alginolyticus*.

N-terminal amino acid sequencing was performed in order to compare the 35.6 kDa OMP with known proteins. The sequence of the first 27 amino acid residues

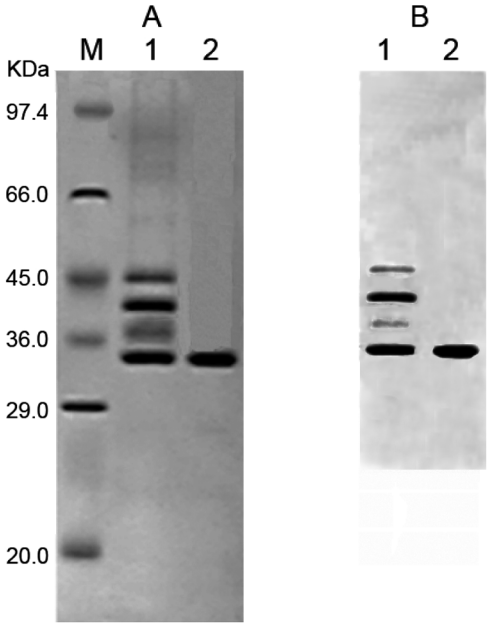

Fig. 1. *Vibrio alginolyticus*. (A) SDS-PAGE profile of crude outer membrane proteins (OMPs) and major outer membrane protein OmpU, and (B) western blot analysis with anti-*V. alginolyticus* serum. Lanes: M, molecular mass markers; 1, crude OMPs; 2, the isolated OmpU

from the N-terminal end of the protein is ATV YKD GGT ELL VGG RVE FRG DFI GSD. A search of the GenBank and BLAST sequence database revealed 92, 88, 88 and 88% similarity with the OmpU proteins of *Vibrio harveyi* HY01, *V. parahaemolyticus* AQ3810, *V. alginolyticus* 12G01, and *V. campbellii* AND4, respectively (Fig. 2).

35.6 kDa-Omp of *V. alginolyticus* HY9901	ATV YDG GTE KLL VGG RVE FRG DFI GSD
OmpU of *V. harveyi* HY01	ATV YKD GGT ELK VGG RVE FRG DFI GSD
OmpU of *V. parahaemolyticus* AQ3810	ATV YKN DGT ELK VGG RVE FRG DFI GSD
OmpU of *V. alginolyticus* 12G01	ATV YKN DGT ELK VGG RVE FRG DFI GSD
OmpU of *V. campbellii* AND4	ATV YKD DST ELK VGG RVE FRG DFI GS-
OmpU of *V. vulnificus* CMCP6	ATV YKT DGT ELK IGG RVE FRG DFI GTD

Fig. 2. Similarity from BLAST sequence database among N-terminal sequences of the 35.6 kDa OMP of *Vibrio alginolyticus* and other *Vibrio* spp. OmpU proteins: *V. harveyi* HY01 (GenBank no. ZP_01987812, 92% sequence homology), *V. parahaemolyticus* AQ3810 (GenBank no. ZP_01990045, 88% sequence homology), *V. alginolyticus* 12G01 (GenBank no. ZP_01262703, 88% sequence homology), *V. campbellii* AND4 (GenBank no. ZP_02195095, 88 % sequence homology) and *V. vulnificus* CMCP6 (GenBank no. NP 762536, 81 % sequence homology

Immunoreactivity of the OmpU of *Vibrio alginolyticus*

To assess the role of the OmpU in the pathogenicity of *Vibrio alginolyticus* during natural infection, western blotting of crude OMPs and the isolated OmpU with rabbit sera against *V. alginolyticus* was conducted and showed recognition of the OmpU (Fig. 1B, Lanes 1 and 2).

Analysis of serum antibody levels

The antibody production against the OmpU of *Vibrio alginolyticus* by *Lutjanus erythropterus* is shown in Fig. 3. The results show that a specific antibody titer against OmpU was detected in all sera of the vaccinated fish. During Weeks 1 to 10 post-vaccination, $\log_2$(antibody titers) in the sera of the vaccinated group all reached above 5.0, and the maximum reached 14.0, while that of the control group was only 1.0 to 3.0 at all times. Comparison of the means of $\log_2$(antibody titers) between the vaccinated and control groups revealed a statistically significant difference ($p < 0.01$).

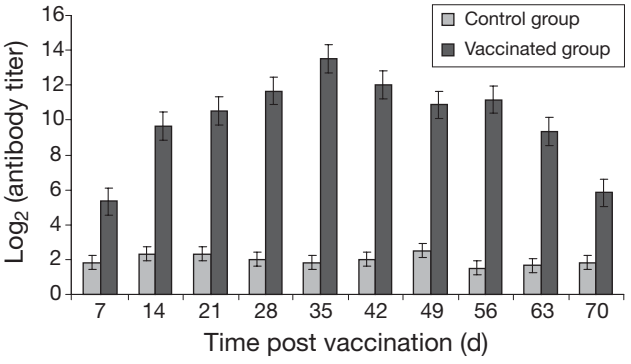

Fig. 3. Analysis of serum antibody levels against OmpU of *Vibrio alginolyticus* in *Lutjanus erythropterus*. Each bar represents the $\log_2$ value of the highest dilution of the serum pool when (OD_{450} value of the inspected sera)/(OD_{450} value of the negative serum) > 2.9. Values are significantly different from the control ($p < 0.01$)

Efficacy of the OmpU vaccine

The cumulative mortality of *Lutjanus erythropterus* after challenge with virulent *Vibrio alginolyticus* is shown in Table 1. The fish were immunized with the OmpU vaccine and obtained a high level of protection. The results showed that the fish mortality rate was significantly lower in the vaccinated groups than in the control group after challenge with *V. alginolyticus* ($p < 0.01$). The tested fish in the control group began to die on Day 2, with a sudden increase in the daily death rate on Days 3 to 6, then a decrease until Day 14. The dead fish showed typical signs of vibriosis, including anorexia, skin ulcers and necrosis of the tail fin and scales and no pathogen other than *V. alginolyticus* was isolated. The RPS value for the OmpU vaccine was 96.43.

Table 1. *Lutjanus erythropterus* infected with *Vibrio alginolyticus*. Cumulative mortality after challenge

Days after challenge	Vaccinated group		Control group	
	No. of dead fish	Mortality rate (%)	No. of dead fish	Mortality rate (%)
1	0	0	0	0
2	0	0	4	7.02
3	0	0	20	35.09
4	0	0	32	56.14
5	0	0	41	71.93
6	1	1.75	46	80.70
7	1	1.75	47	82.46
8	1	1.75	49	85.96
9	1	1.75	51	89.47
10	1	1.75	52	91.23
11	2	3.52	54	94.74
12	2	3.52	56	98.25
13	2	3.52	56	98.25
14	2	3.52	56	98.25

DISCUSSION

OMPs are important surface materials for bacterial infections. They have multiple functions and roles that help bacteria survive, such as acting as antennae for environmental signals, adhesion for colonization and also as invasion mediators (Beher et al. 1980, Sperandio et al. 1995, Jung et al. 2005). We describe the isolation and characterization of an immunogenic protein from the outer membranes of *Vibrio alginolyticus*. The OMPs of Gram-negative bacteria are immunologically important because of their accessibility to the host defense system (Hamid & Jain 2008, Yang et al. 2008). However, there is only relatively limited information available about the potential of OMPs to confer protection against *V. alginolyticus*. The gel excision method was used as the approach of choice for isolation of OmpU because of the relative ease of working with the OMP fraction recovered from the gels. This method has yielded good results in our laboratory.

Some of the important attributes of a candidate molecule for vaccine development are that it should be highly conserved among various members of the same genus, should be expressed on the surface of pathogens so that antigen-presenting cells can easily recognize them, and should be immunogenic (Khushiramani et al. 2007). Vaccination with OmpK and OmpW has been shown to be effective against *Vibrio* infections in fish (Qian et al. 2007, Li et al. 2008). In the present study, the N-terminal amino acid sequence of the OmpU of *V. alginolyticus* was found to have very high homology with those of other *Vibrio* (≤92%) by BLAST analysis. When rabbit polyclonal antibodies against *V. alginolyticus* were tested in western blot with crude OMPs and the isolated OmpU, a strong reaction was observed between the rabbit antiserum and OmpU. This proves that the OmpU of *V. alginolyticus* retained natural antigenicity. Therefore, we presume the OmpU could be used as a potential vaccine candidate.

The present study suggested that a strong antibody response was induced and significant protection was achieved by vaccination with the OmpU of *Vibrio alginolyticus* in *Lutjanus erythropterus*. Some studies have also reported that native or recombinant OMPs can induce protective immunity in aquatic animals (Mao et al. 2007, Li et al. 2008, Qian et al. 2008). The native N-lauroylsarcosine-extracted OMP of *Edwardsiella ictaluri* could partially protect channel catfish from ulcer disease (Bader et al. 2004). Khushiramani et al. (2007) reported that the recombinant OmpTS of *Aeromonas hydrophila* provided protection for Indian major carp *Labeo rohita* with a RPS of 57. Mao et al. (2007) reported that the recombinant OmpK of *V. parahaemolyticus* provided protection for large yellow croaker with a RPS of 90. Recently, it was shown that the recombinant OmpK of *V. harveyi* could provide protection for orange-spotted grouper with a RPS of 100 (Li et al. 2008). In our study, *L. erythropterus* vaccinated with the isolated OmpU of *V. alginolyticus* were protected with a RPS of 96.43. The different RPS values reported for outer membrane fraction vaccines probably result from the different amino acid sequences, the different virulence of the bacteria and the different immune tolerance in the tested animals.

Vibrio alginolyticus is one of the main *Vibrio* pathogens in marine animals, and results in serious economic losses. It is, therefore, desirable to develop an effective vaccine against the disease. Vaccination with OMPs has been shown to be effective against infection by *V. vulnificus*, *V. anguillarum* and *V. harveyi* in fish (Jung et al. 2005, Kumar et al. 2007, Li et al. 2008). The present study indicated that the isolated OmpU is an effective vaccine candidate against infection by *V. alginolyticus*. OmpU gene cloning and over-expression is ongoing.

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