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Original Article

Enhancement of protective immunity in European eel (*Anguilla anguilla*) against *Aeromonas hydrophila* and *Aeromonas sobria* by a recombinant *Aeromonas* outer membrane protein

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To develop a vaccine, which can simultaneously prevent the diseases caused by various pathogenic bacteria in fish, we try to find a conserved outer membrane protein (OMP) antigen from different bacterial pathogens. In this study, an OMP fragment of 747 bp (named as Omp-G), which was highly conserved in seven *Aeromonas* OMP sequences from the NCBI database, was amplified by PCR from one *Aeromonas sobria* strain (B10) and two *Aeromonas hydrophila* strains (B27 and B33) with the designed specific primers. The sequence was cloned into pGEX-2T (6 × His-tag) vector, expressed in *Escherichia coli* system, and then the recombinant protein (named as rOmp-G) was purified with nickel chelating affinity chromatography. The purified rOmp-G showed a good immunogenicity in rabbits and well-conserved characteristics in these three pathogens by enzyme-linked immunosorbed assay. Furthermore, the rOmp-G also showed good immunogenicity in eels (*Anguilla anguilla*) for eliciting significantly increased specific antibodies ($P < 0.01$), and providing higher protection efficiencies ($P < 0.05$) after the pathogens challenge. The values of the relative percent survival in eels were 70% and 50% for two *A. hydrophila* strain challenge, and 75% for *A. sobria* strain challenge. This is the first report of a potential vaccination in eels that simultaneously provide protectiveness against different *Aeromonas* pathogens with a conserved partial OMP.

Keywords outer membrane protein; immunogenicity; *Aeromonas sobria*; *Aeromonas hydrophila*; *Anguilla anguilla*

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Introduction

Eel (*Anguilla* spp.) is one of the most important economic and cultivated fish species in China [1], while diseases caused by pathogenic bacteria often breakout and spread in eel industry and sometimes cause large economic losses [2]. Except for the chemotherapy, there is no efficient way to prevent the diseases at present, but the chemotherapy tends to cause drug resistance of pathogens, environmental pollutions, and drug residue in eel products [3]. Recently, vaccination has been proved to be a safe and effective solution in preventing bacterial infections in fish [4], however, the cultivated fish are often subject to various pathogenic bacteria, and the administration of several vaccines at the same time is tedious and uneconomical. A polyvalent vaccine that can prevent more than one pathogen is one of the most attractive ways to solve this problem. Some polyvalent vaccines prepared with the whole bacteria cells have been used in fish [5,6], while the immunological processes such as antigenic competition or antigen-induced suppression always affected the vaccines efficiency. Therefore, the vaccine prepared with the conserved protective antigen shared by different pathogenic bacteria may provide a promising strategy for controlling the bacterial infections in aquaculture.

Outer membrane proteins (OMPs), as the interface between host and pathogens, located on the surface of Gram-negative bacteria and are easily recognized as foreign substances by the immune system of the hosts [7], which indicates that they are promising vaccine candidates. The OMPs can induce specific bactericidal antibodies [8–10], inhibit the bacterial colonization in hosts [11], and induce cell-mediated immunity [12–14]. Many OMPs from different pathogenic bacteria, such as *Aeromonas salmonicida* [8,15–17], *Aeromonas hydrophila* [9,18–21], *Vibrio parahaemolyticus* [22], *Vibrio alginolyticus* [23], *Vibrio harveyi* [12–13], *Edwardsiella ictaluri* [10], and *Edwardsiella tarda* [14,24], have been identified as effective protective antigens. Some OMPs mixtures extracted from one pathogenic bacterial strain have been proved to

provide cross protection for fish against several other bacterial strains or species [15,20], suggesting that different pathogenic bacterial species may share some similar or conserved OMP antigens. Recently, further studies have shown that different bacterial strains or species share OMPs with the same molecule weight, which have good immunogenicity to the host [16–17,21,24–28]. Moreover, some of these OMPs have been proved to have effective protections for fish against the corresponding bacterial species [16] or other different serotypes strains [17,21,24], which indicates that such OMPs from different pathogens might be used to develop a vaccine for simultaneously preventing the diseases caused by different pathogenic bacteria.

In this study, a conserved OMP nucleotide fragment was cloned from three *Aeromonas* pathogens isolated from eels, and the immunogenicity and conserved characteristics of its recombinant OMP were examined and compared between immunized rabbits and eels. The results showed a potential way to develop a vaccine that can prevent the diseases caused by different bacterial pathogens in aquaculture.

Materials and Methods

Database, bacterial strains, and eels

According to the reported OMPs nucleotide sequences of the fish pathogens, blasts were conducted in NCBI database (GenBank + EMBL + DDBJ + PDB) in order to get the possible conserved OMP nucleotide fragments of different pathogens.

Twenty bacteria strains that had previously been proved to be pathogenic by infection experiments in our laboratory were used to amplify the possible conserved OMP nucleotide fragments by PCR. These strains were isolated from 3 species of eels in different areas of China, and were identified as 12 *A. hydrophila* strains of different serotypes (B11, B12, B13, B21, B26, B27, B29, B33, B34, B35, B36, and B37), 3 *A. sobria* strains (B10, B40, and B41), 2 *A. veronii* strains (B44 and B45), 1 *E. tarda* strain (B09), 1 *Vibrio vulnficus* strain (B43) and 1 *A. caviae* strain (B46) by physiological and biochemical identification [29].

European eels (*Anguilla anguilla*) weighing 30 ± 5 g, without a previous history of diseases, were used for vaccination and infection challenge experiments. The eels were acclimatized for 2 weeks in a 500 L tank before the vaccination experiments. During the experiment, the eels were maintained at the temperature of 28 ± 1°C in 100 L tanks with filtration and recirculation freshwater system, and fed daily with a commercial pellet diet at 1% of eel body weight. As a negative control, eels were kept and fed at the same conditions in an individual 100 L tanks during the experiments.

Screening conserved OMPs from NCBI and from pathogenic bacteria strains

The OMP nucleotide sequences of fish pathogens in NCBI were searched and analyzed by multiple alignments. Seven OMP nucleotide sequences of *A. hydrophila* with a homology more than 97% were chosen as the conserved OMP templates for designing specific primers. Their accession numbers in GenBank are DQ302124.1, CP000462, DQ302125, AF276639, DQ188832, EU925558, and EF189590.1, respectively.

A pair of specific primers was designed based on the most conserved regions (747 bp length) of these seven OMP nucleotide sequences. Forward primer: 5′- CG GGATCC (*Bam*H I) TGGTCCGGCAAGATTGCTC-3′; reverse primer: 5′-CAGAATTC (*Eco*R I) CAGCAGGGT TTCGTCAAGC- 3′). Two restriction sites of *Bam*H I and *Eco*R I were also included (underlined).

Twenty bacterial genomic DNAs were used as the templates to amplify the expected 747-bp conserved OMP nucleotide fragments (named as Omp-G). The PCR reaction was performed in a 20 μl mixture with the designed specific primers and Pfu DNA polymerase (TaKaRa, Dalian, China), and the PCR protocol was one cycle of 94°C for 5 min, 30 cycles of 94°C for 50 s, 62°C for 50 s, and 72°C for 50 s, followed by one cycle of 72°C for 10 min.

Recombinant plasmid construction and sequence analysis

After double-restriction enzyme digestion with *Bam*H I and *Eco*R I (TaKaRa), the Omp-G was ligated to the pGEX-2T (with 6 × His-tag) expression plasmid (a modified plasmid, kind gift from Key Laboratory of Marine Biogenetic Resources, Third Institute of Oceanography, Xiamen, China). The recombinant pGEX-2T-Omp-G (6 × His-tag) plasmid was transformed into *E. coli* BL21 (Invitrogen, Carlsbad, USA). The positive *E. coli* BL21 harboring the pGEX-2T-Omp-G (6 × His-tag) was selected by *ampicillin* resistance. The recombinant plasmid was identified by restriction enzymes cleavage (*Bam*H I and *Eco*R I) and DNA sequencing.

The cloned Omp-G sequence was submitted to NCBI database. The nucleotide sequences were compared with GenBank/EMBL/DDBJ/PDB database using the BLAST program from NCBI. The homology between the deduced amino acid sequence of this Omp-G and other known OMPs in the NCBI database was compared using BLAST program and DNAMAN 5.0 software.

Expression and purification of the recombinant Omp-G

The *E. coli* BL21 containing the expected plasmid was cultured with shaking at 220 rpm, overnight at 37°C, diluted

1:100 (V/V), and cultured in fresh Luria Broth with *ampicillin* (50 μg/ml) until the optical density at 600 nm (OD_{600}) reached approximately 0.6, then induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma, St. Louis, USA) for 7 h at 37°C. To confirm the expression, the induced bacteria were lysed and separated by SDS-PAGE (12%), followed by Coomassie blue staining. The relative quantity of the expressed recombinant protein (named as rOmp-G) was determined by thin layer scanning.

Owning to the six histidines at the N terminal, the rOmp-G was purified by Ni Sepharose 6 Fast Flow (Amersham Biosciences, Uppsala, Sweden) with ÄKTApurifier-100 (GE Healthcare Life Science, Piscataway, USA) according to the manual. Briefly, the *E. coli* BL21 expressing the rOmp-G were harvested by centrifugation, then washed and suspended in buffer A [20 mM phosphate buffered saline (PBS), 500 mM NaCl, pH 7.4]. The cell suspension was disrupted by sonication (300 W, 100 × 10 s) in an ice bath and centrifuged at 12 000 × g for 20 min at 4°C. The precipitate containing the rOmp-G was washed in buffer A containing 2 M urea, and dissolved in buffer I (20 mM PBS, 500 mM NaCl, 20 mM imidazole, 8 M urea, pH 7.4), then was loaded on the Ni Sepharose 6 Fast Flow. The column was washed with buffer A and then with buffer I, and finally eluted with the continuous imidazole gradient from 20 mM to 500 mM in 20 mM PBS, pH 7.4, containing 500 mM NaCl and 8 M urea. Finally, the rOmp-G protein was refolded by dialyzing against buffer A with decreasing concentrations of the urea. The rOmp-G concentration was determined by Bradford method [30], and then the rOmp-G was aliquoted and stored at −80°C before use.

Preparation of rabbit anti-rOmp-G-sera

In order to analyze the immunogenicity of the rOmp-G in rabbits, rabbit anti-rOmp-G sera (named as S-rOmp-G) were prepared according to Kawai *et al*. [24]. Rabbits (New Zealand white rabbits, Shanghai Laboratory Animal Center, Shanghai, China) weighing 2.5 ± 0.5 kg, were immunized with the rOmp-G (200 μg per rabbit) by intramuscular injection at 7 day intervals for 4 weeks consecutively. The first dose was administered with Freund's complete adjuvant, while the subsequent doses were given with Freund's incomplete adjuvant. Three days after the final booster injection, the rabbits were bled and the blood was allowed to clot at room temperature for 1 h and then stored at 4°C overnight. The separated anti-sera obtained by centrifugation were aliquoted and stored at −80°C till use. Specific antibody titer of the S-rOmp-G was determined with indirect ELISA. The serum of pre-immunized rabbit was sampled as control.

Preparation of rabbit anti-pathogens sera

In order to analyze the conserved characteristics of the rOmp-G in the pathogens, rabbit anti-sera against those *Aeromonas* pathogens (B10, B27, and B33), which were amplified for the conserved OMP by PCR were also prepared according to Kawai *et al*. [24], and were named as S-B10, S-B27, and S-B33, respectively. Formalin-killed cells (FKC) of *Aeromonas* B10, B27, and B33 were used as the immunizing antigens. The FKC were prepared by inactivating the cultured bacterial cells of three *Aeromonas* pathogens with a final concentration of 0.5% formalin at 56°C for 3 h. The FKC were collected by centrifugation and suspended in PBS. New Zealand white rabbits were immunized with the FKC preparations (1×10^8 CFU per rabbit) by intramuscular injection. After 4 weeks, the booster dose injections were performed at 14-day intervals for three times. The first dose was administered with Freund's complete adjuvant, while the subsequent doses were given with Freund's incomplete adjuvant. The rabbit anti-sera were separated as described above.

Preparation of rabbit anti-eel-IgM sera

In order to gain the secondary antibody for evaluating the specific antibody titer of eels by indirect ELISA, the rabbit anti-eel sera (S-eel-IgM) were prepared. IgM fraction was obtained from the serum of eels (*A. anguilla*) by gel filtration and ion exchange chromatography according to Li *et al*. [31]. The IgM fraction was mixed with Freund's incomplete adjuvant and injected subcutaneously into rabbits. The S-eel-IgM was obtained as described above.

Determination of the antibody titers by indirect ELISA analysis

The specific antibody titer against the rOmp-G in the S-rOmp-G was evaluated by indirect ELISA [24]. Briefly, 96-well ELISA plates were coated overnight at 4°C with the rOmp-G (10 μg/ml) in 100 μl of carbonate coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6), then washed three times with PBS containing 0.05% Tween-20 (PBST). The ELISA plates were then blocked with 5% skimmed milk in PBS for 2 h at 37°C, followed by washing with PBST for three times. Subsequently, 2-fold serial dilutions of the S-rOmp-G (from 1:50 to 1:51200) or control sera (from 1:50 to 1:51200) were added into each well of the plates and incubated for 90 min at 37°C, followed by washing with PBST for three times. The goat anti-rabbit IgG conjugated with HRP (1:2000; Sigma) was added into each well, and the ELISA plates were incubated for 1 h at 37°C before washing with PBST for three times again. Then 50 μl of *O*-phenylenediamine substrate buffer was added into each well and the ELISA plates were incubated for 10 min at 37°C. Fifty microliters of H_2SO_4 (2 M) was added to stop the reaction. The absorbance of each

well was measured by μQuant spectrophotometer (Bio-TEK Instruments, Inc., Winooski, USA) at 492 nm. Results were considered positive if the absorbance was at least double of the control sera, and the antibody titers were scored as the highest positive dilution.

The specific antibody titers of the prepared S-B10, S-B27, or S-B33 against the *Aeromonas* pathogens of B10, B27, or B33 were evaluated by indirect ELISA assay. The conservation of the rOmp-G in B10, B27, and B33 was also evaluated by indirect ELISA assay. The prepared S-rOmp-G was reacted with one of the three *Aeromonas* strains (B10, B27, and B33), and the rOmp-G was also reacted with one of the three anti-sera of S-B10, S-B27, and S-B33. The reaction between the rGST-His-tag protein and the S-rOmp-G, S-B10, S-B27, or S-B33 was assessed as control.

Eel vaccination, challenge, and survival rates calculation

The experimental European eels ($n = 156$) were divided into four tanks. Each tank was stocked with 39 eels. Each eel from two tanks was immunized intraperitoneally with 30 μg of rOmp-G dissolved in 100 μl PBS (treatment group), and the eels from the other two tanks were injected intraperitoneally with 100 μl PBS (control group). The same administration was done again after 10 days. On the 14th, 21st, and 28th day after the first immune injection, six eels from treatment group (three eels of each tank) and six eels from control group (three eels of each tank) were bled, and the sera were prepared for specific antibody titer detection.

On the 29th day after the first administration, the remaining eels from each tank ($n = 30$) were regrouped into three tanks, then the eels of each tank were challenged by intraperitoneal injection with one of the three live *Aeromonas* strains (B10, B27, and B33), respectively. The challenge dose was 2×10^7 CFU per fish according to the LD_{50} value of the strains [29]. The cumulative mortality and clinical signs were daily recorded for 28 days post-challenge, and the dying fish was autopsied to isolate the pathogenic bacteria. The mortalities of eels challenged by the same pathogenic bacteria were compared between the treatment group and the control group, and the relative percent survival (RPS) was also compared using the following equation [32]:

$$\mathrm{RPS} = \frac{1 - \text{mortality in vaccinated fish}\,\%}{\text{mortality in control fish}\,\%} \times 100.$$

Immunogenicity analysis of the rOmp-G in eels by ELISA detection

Sera that collected from six eels from treatment group and control group on the 14th, 21st, or 28th day after the first administration were used for specific antibody titer determination by ELISA [14]. The 2-fold serial dilutions of the eel sera (from 1:50 to 1:12800) were added to the wells of ELISA plates, which were firstly coated with rOmp-G (10 μg/ml), then with the S-eel-IgM (1:2000) and the HRP-conjugated goat anti-rabbit IgG (1:5000; Invitrogen) were added sequentially. Absorbance of each well was measured by a μQuant spectrophotometer at 492 nm. Results were considered positive if the absorbance was at least double of the control sera, and antibody titers were scored as the highest positive dilution. Six independent assays were performed, and the results were presented as mean ± SD. The significant difference was calculated by Student's test at the level of $P < 0.01$.

Western blot analysis

The conservation of the rOmp-G in the three *Aeromonas* strains of B10, B27, and B33 was also analyzed by western blot using eel anti-sera that come from the survival eels after the experimental infections with these three *Aeromonas* strains [22]. The rOmp-G was separated on 12% SDS-PAGE gel, and transferred to polyvinylidene-fluioride membranes (Amersham Biosciences). The membranes were blocked with 5% (w/v) skimmed milk in tris-buffered saline (TBS) (150 mM NaCl, 20 mM Tris-base, pH 7.4) for 1 h, and then incubated with the serum of survival eels after experimental infections (1:200 diluted in TBS) at 37°C for 1.5 h. The membranes were washed three times with TBST (TBS with 0.1% Tween-20) before they were incubated with S-eel-IgM (1:2000) at 37°C for 1.5 h, followed by washing with TBST for three times. The membranes were finally incubated with TBST containing goat anti-rabbit IgG conjugated with HRP (1:5000) for 1 h. The membranes were visualized with DAB for 30 s. As a control, the serum sampled from the negative eel was also used for western blot assay with the same protocol.

Results

Molecular cloning and sequence analysis of the Omp-G

Using the designed specific primers, the expected OMP fragment with the length about 747 bp (named as Omp-G) was amplified from one *A. sobria* (B10; lane 13, **Fig. 1**) and two different serotypes of A. hydrophila (B27; lane 6, **Fig. 1**, and B33; lane 8, **Fig. 1**). The PCR results from all other pathogenic bacterial species like *A. veronii*, *A. caviae*, *E.tarda* and *V. vulnficus* included in this study were negative (**Fig. 1**). After constructing the recombinant plasmid of pGEX-2T-Omp-G (6 × His-tag), we sequenced the nucleotide sequence of the Omp-G and submitted it to NCBI GenBank (accession no. FJ437030). The nucleotide

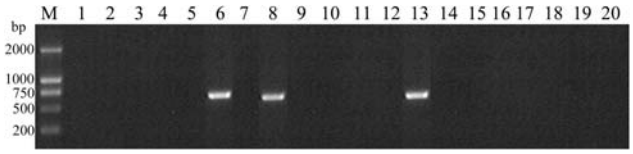

Figure 1 PCR amplification of the expected OMP fragment of 747 bp (named as Omp-G) from 20 strains of pathogenic bacteria isolated from cultivated eels The Omp-G was amplified from one *Aeromonas sobria* (B10; lane 13) and two different serotype of *A. hydrophila* strains (B27; lane 6, and B33; lane 8). M, molecular marker (200 bp ladder); 1–12, 12 *A. hydrophila* strains belonging to different serotypes (B11, B12, B13, B21, B26, B27, B29, B33, B34, B35, B36, B37); 13–15, three *A. sobria* strains (B10, B40, B41); 16–17, two *A. veronii* strains (B44, B45); 18–20, *Edwardsiella tarda* (B09), *Vibrio vulnficus* (B43) and *A. caviae* (B46) strains.

sequence and deduced amino acid sequence of the Omp-G were shown in **Fig. 2**. In order to find out the homology between the Omp-G and other known OMPs in the NCBI database, BLAST with this Omp-G was performed. The results showed that this protein (encoded by the Omp-G) had 100% homology with an OMP of *A. hydrophila* ATCC 7966 (YP867290.1) and a major adhesin Aha1 of *A. hydrophila* (ABC54614.1). The function of these two proteins so far is unknown. This *Aeromonas* Omp-G fragment protein was also found to have high homology (overall identities from 77% to 99%) with several other OMPs from different *A. hydrophila*, *A.veronii*, and *A. salmonicida* (**Table 1**), which indicated that this Omp-G was conserved in different *Aeromonas* genus. According to the prediction by Hidden Markov model method, this *Aeromonas* Omp-G fragment protein was classified into porin protein family, suggesting that it had good immunogenicity and was a suitable candidate for developing vaccine against bacterial infections.

Expression and purification of the rOmp-G

After the *E. coli* BL21 was induced with IPTG, a protein band about 55.2 kDa (the rOmp-G) was found in the positive *E. coli* BL21 (**Fig. 3**, lane 1). This fusion protein (55.2 kDa) was composed of the Omp-G protein (28.2 kDa) and the rGST-His-tag (27 kDa). In control, no such protein was found either in the induced negative *E. coli* BL21 (**Fig. 3**, lane 2) or in the non-induced positive *E. coli* BL21 (**Fig. 3**, lane 3). The over-expressed rOmp-G was about 20% of the total proteins of the *E. coli* BL21 (**Fig. 3**, lane 1) when analyzed by thin layer scanning. The rOmp-G was only found in the precipitate (**Fig. 3**, lane 5) of the induced positive *E. coli* while not in its supernatant (**Fig. 3**, lane 4), which showed that the rOmp-G existed in the inclusion body form.

The rOmp-G was subsequently purified by ÄKTApurifier-100 system under denaturing condition, and two peaks of a flow through (**Fig. 4**, Peak 1) and an eluted pool (**Fig. 4**, Peak 2) appeared when eluted by nickel chelating affinity chromatography. The eluted pool peak (**Fig. 4**, Peak 2) appeared when the concentration of imidazole reached 300 mM. When analyzed by SDS-PAGE, the fractions from the eluted pool peaks showed only a single band with molecular mass of about 55.2 kDa (**Fig. 3**, lane 8), which has a same molecular mass with that of the protein appeared in the induced positive BL21 (**Fig. 3**, lane 1) and that in precipitates (**Fig. 3**, lane 5). The fraction from the flow-through peak did not show such a distinct

```
1       TGGTCCGGCAAGATTGCTCTGAACAACACCTGGTCCGGTATCGCCAAGACCGAGTGGCAAGTTTCTGCTGAAAAC
1        W  S  G  K  I  A  L  N  N  T  W  S  G  I  A  K  T  E  W  Q  V  S  A  E  N

76      TCCGCCAACAAGTTTGACTCCCGTCACATCTACGTTGGTTTCGACGGCACCCAATACGGCAAGATCATCTTCGGT
26       S  A  N  K  F  D  S  R  H  I  Y  V  G  F  D  G  T  Q  Y  G  K  I  I  F  G

151     CAGACCGATACCGCGTTCTACGACGTGCTGGAACCGACCGATATCTTCAACGAGTGGGGTGATGCAGGTAACTTC
51       Q  T  D  T  A  F  Y  D  V  L  E  P  T  D  I  F  N  E  W  G  D  A  G  N  F

226     TATGACGGTCGTCAAGAAGGTCAGGTTATCTACTCCAACACCTACGGTGGCTTCAAAGGCAAACTGTCCTATCAG
76       Y  D  G  R  Q  E  G  Q  V  I  Y  S  N  T  Y  G  G  F  K  G  K  L  S  Y  Q

301     ACCAACGACGACAAAGCCGTCAAGGTTACTGACGTAGGTCAGGGTATCAAAGAAACTGCCGTTTACGGTGCCGAT
101      T  N  D  D  K  A  V  K  V  T  D  V  G  Q  G  I  K  E  T  A  V  Y  G  A  D

376     GTGAAGCGTAACTACGGTTATGCCGCTGCTGCCGGTTATGACTTCGACTTCGGTCTGGGCCTGAACGCAGGTTAC
126      V  K  R  N  Y  G  Y  A  A  A  A  G  Y  D  F  D  F  G  L  G  L  N  A  G  Y

451     TCCTACTCCGATCTGGAAAACACCAAGACCAACAACACTGGTGACAAGAAAGAGTGGGCACTGGGTGCACACTAC
151      S  Y  S  D  L  E  N  T  K  T  N  N  T  G  D  K  K  E  W  A  L  G  A  H  Y

526     GCCATCAACGGTTTCTACTTCGCTGGTGTTTATACTCAGGGTGACCTGAGCTACGACAACACCACCGGTGGTGAC
176      A  I  N  G  F  Y  F  A  G  V  Y  T  Q  G  D  L  S  Y  D  N  T  T  G  G  D

601     AAGGACAAGGGCCGTGGTTACGAGCTGGCAGCTTCCTACAACGTTGATGCCTGGACCTTCCTGGCTGGCTACAAC
201      K  D  K  G  R  G  Y  E  L  A  A  S  Y  N  V  D  A  W  T  F  L  A  G  Y  N

676     TTCAAAGAAGCAAAAGTCAACTCCAATACTGCTGGTGCAGAGTACAAAGACCTGCTTGACGAAACCCTGCTG
226      F  K  E  A  K  V  N  S  N  T  A  G  A  E  Y  K  D  L  L  D  E  T  L  L
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Figure 2 Nucleotide sequence and deduced amino acid sequence of the expected OMP fragment of 747 bp (named as Omp-G) from those three *Aeromonas* strains of B10, B27, and B33 The sequence has been submitted to the GenBank database with the accession number *FJ437030*.

Table 1 Homology between the amino acid sequence of the expected OMP fragment of 747 bp (named as Omp-G) and other known OMPs analyzed by the BLAST program

Descriptions	Accession number	Homology (%)
Omp-G protein	ACJ61007.1	100
OMP [*A. hydrophila* ATCC 7966]	YP 857290.1	100
Major adhesin Aha1 [*A. hydrophila*]	ABC54614.1	100
OMP [*A. hydrophila*]	AAF87725.2	99
Major adhesin Aha1 [*A. hydrophila*]	ABC 54617.1	99
Major adhesin Aha1 [*A. hydrophila*]	ABC 54615.1	99
Major adhesin [*A. hydrophila*]	ACH53196.1	99
Adhesin [*A. hydrophila*]	ABA28756.1	98
Major adhesin Aha1 [*A. hydrophila*]	AAN84549.1	95
Major adhesin protein [*A. hydrophila*]	ABM69030.1	93
Major adhesin protein [*A. hydrophila*]	ACB0657.1	92
Major adhesin Aha1 [*A. hydrophila*]	ABC54616.1	80
OMP porin II [*A. hydrophila*]	AAD56398.1	79
Omp38 protein precursor [*A. veronii*]	AAP40343.2	78
Porin II (OmpK40) [*A. salmonicida*]	YP 001141380.1	77

This Omp-G protein was highly conserved in different *Aeromonas* pathogens with the identities above 77%.

band (**Fig. 3**, lane 7), indicating that most of the rOmp-G bound to the column specifically.

Immunogenicity of the rOmp-G

Aromatic amino acids were rich in the conserved sequence of the OMP (>16%), and involved in forming the secondary structures of helix, sheet, or coil (SOMPA method, http://www.expasy.org). This recombinant protein showed good antigenicity and hydrophicity when analyzed by Jomeson-Wolf and Plot-Kyte-Doolittle methods with DNASTAR software. In addition, no cysteine was found and therefore no disulfide linkage existed in the rOmp-G.

The recombinant protein (rOmp-G) was highly immunogenic in rabbits and eels. In rabbits, after the 3rd booster dose, the antibody titer against the rOmp-G reached 1:6400, but the reaction titer against the rGST-His-tag protein was

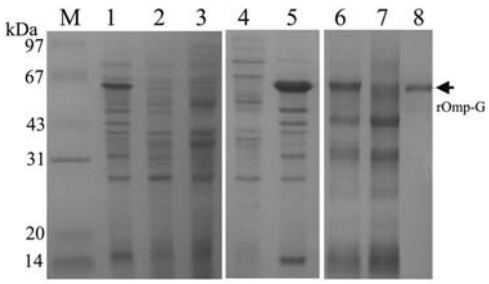

Figure 3 SDS-PAGE analysis of the expression and purification of *E. coli* BL21 harboring the recombinant pGEX-2T-Omp-G (with His-tag) The rOmp-G was over-expressed and the purified protein showed a single band with about 55.2 kDa in SDS-PAGE gel. M, low molecular weight marker; 1, recombinant *E. coli* BL21 with IPTG induction; 2, non-recombinant *E. coli* BL21 cells with IPTG induction; 3, recombinant *E. coli* BL21 without IPTG induction; 4, the supernatants of whole-induced recombinant *E. coli* BL21; 5, the precipitates of whole-induced recombinant *E. coli* BL21; 6, starting loaded protein; 7, flow-through protein (Peak 1); 8, eluted pool protein (Peak 2).

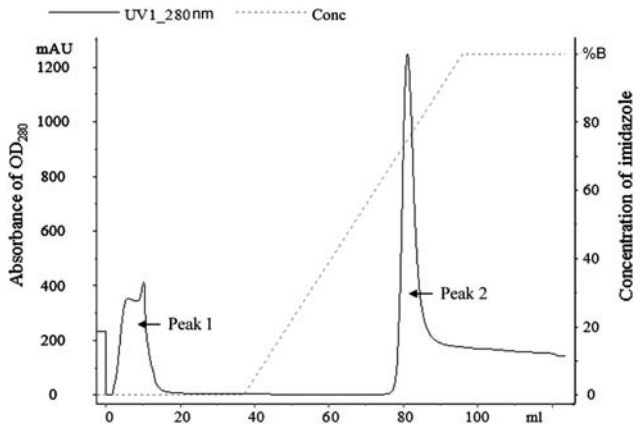

Figure 4 Profile of the purification of the rOmp-G by Ni Sepharose 6 Fast Flow with ÄKTA purifier-100 Peak 1, flow-through; Peak 2, eluted pool.

only 1:400 (**Table 2**). These results suggested that the rGST-His tag did not affect the immunogenicity and specificity of the rOmp-G in rabbits. In eels, on the 14th, 21st, and 28th day after the first immunization with the rOmp-G, the specific antibodies against the rOmp-G were also detected. Significantly high ($P < 0.01$) antibody response was detected in the sera of treatment group ($n = 6$) immunized with the rOmp-G, when compared with those of the control group injected with PBS. Moreover, the antibody titers in the treatment group reached 1:800 on the 14th day and the 21st day, and 1:1600 on the 28th day after the immunization (**Fig. 5**). These results suggested that the rOmp-G also had good immunogenicity in eels.

Conserved characteristics of the rOmp-G in the three pathogens

The prepared anti-sera of S-B10, S-B27, and S-B33 were used to analyze the conserved characteristics of the

Table 2 Specific antibodies titers and cross-reaction titers of rabbit anti-sera measured by indirect ELISA analysis

Antigen	Titers of rabbit anti-sera			
	S-rOmp-G	S-B10	S-B27	S-B33
rOmp-G	1:6400	1:400	1:400	1:1600
B10	1:800	1:6400	–	–
B27	1:1600	–	>1:51200	–
B33	1:800	–	–	1:25600
rGST-His	1:400	<1:50	<1:50	<1:50

The specific antibody titer of S-rOmp-G was 1:6400 against the rOmp-G, and the reaction titers against B10, B33, and B27 were 1:800, 1:800, and 1:1600 respectively. And the reaction titers between S-B10, S-B27 or S-B33 and the rOmp-G were 1:400, 1:400 or 1:1600, respectively. It indicated that the rOmp-G had a good immunogenicity in rabbits, and kept the conserved epitopes in these three *Aeromonas* pathogens.

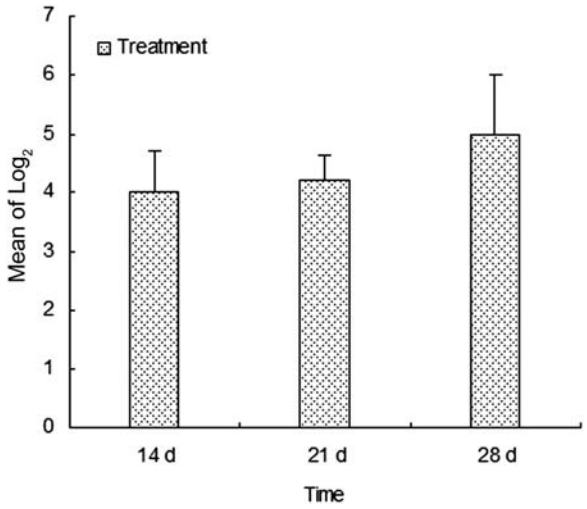

Figure 5 The rOmp-G antibody titers of the treatment eels detected by ELISA The treatment group showed significantly increased specific antibodies against the rOmp-G compared with those of the control group ($P < 0.01$). The control sera were not detected background titers against the rOmp-G at the serum dilution from 1:50 to 1:12800, so the titers in control groups did not appear in the graphs. Each column represents mean $\log_2$ value $\pm$ SD of the highest dilution/50 of the serum because of the least serum dilution was 1:50 ($n = 6$).

rOmp-G in the three pathogens B10, B27, and B33 by indirect ELISA. The results showed that the rOmp-G was all recognized by these three rabbit anti-sera, and the reaction titers were 1:400 for S-B10 and S-B27, and 1:1600 for S-B33 (**Table 2**). In the control group, the rGST-His tag protein did not show significant agglutinations with these three rabbit anti-sera of S-B10, S-B27, and S-B33 (**Table 2**). These results suggested that the rOmp-G kept the conserved epitopes of these three *Aeromonas* strains, and therefore involved in their immunogenicity. At the same time, the reaction titers of the S-rOmp-G against B10, B27, or B33 were 1:800, 1:800, and 1:1600, respectively (**Table 2**), indicating that the antibody against the rOmp-G could recognize the conserved natural antigen-determinates on the cell surface of these three *Aeromonas* pathogens.

The conserved characteristics of the rOmp-G were also proved by western blot using the eel anti-sera against B10, B27, and B33. The 55.2 kDa band (the rOmp-G) was detected with all the sera of the eels that were challenged with these three *Aeromonas* strains (**Fig. 6**, lanes 1–3). But no such band was detected when the sera of the control eel was used (**Fig. 6**, lane 4). This result indicated that the Omp-G protein was one of the important conserved antigens of these three *Aeromonas* pathogens to eels.

Protective immunogenicity of the rOmp-G in eels

To test the protective immunogenicity of the rOmp-G in eels, eels were immunized with the rOmp-G for 28 days, and then followed by the challenge with the three *Aeromonas* strains of B10, B27, or B33. As expected, the immunized eels were significantly protected from the infections with those challenged bacterial strains as compared with the control groups. The RPS values are 75% for B10 challenge [**Fig. 7(A)**], 50% for B27 challenge [**Fig. 7(B)**], and 70% for B33 challenge [**Fig. 7(C)**]. In addition, the challenged bacteria were isolated from the dying eels after the challenge experiment. In the negative control, no eels were dead or sick during the experiment. All these results showed that the rOmp-G was a successful vaccination in eels against those three challenged *Aeromonas* pathogens.

Discussion

OMPs have been proved to be distributed extensively in various pathogenic bacterial strains or species [15–17,20–21,24–28], which makes them to be the potential conserved antigen candidates to explore polyvalent vaccine in fish. Recently, the antigenic cross-reactivity and cross-protection have also been reported in some OMPs, for instance, a conserved 43-kDa OMP from *A. hydrophila* confers significant protection to fish against two different serotypes of *A. hydrophila* challenge [21], and a conserved 37 kDa OMP from *E. tarda* significantly provides protection against the infections by three different serotypes of *E. tarda* in fish [24]. Up to now, there are no reports about whether a recombinant conserved OMP can provide protection against different pathogenic species. In recent years, an OMP nucleotide (ompTS) has been amplified from two *Aeromonas sorbia* strains, one *A. hydrophila* strain and one *A. caviae* strain [33], and an effective protection of the recombinant ompTS against the *A. hydrophila* strain has been proved, while protection against other two species has

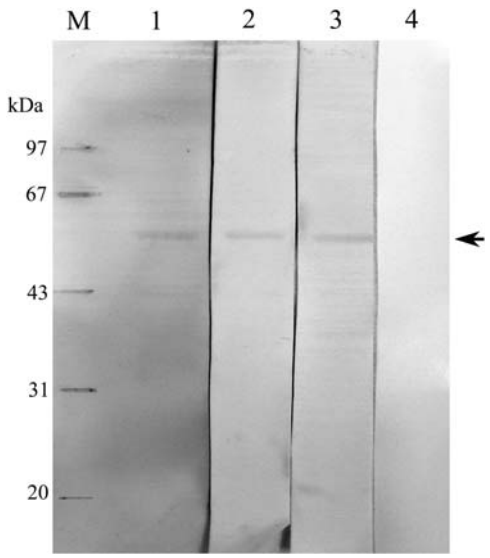

Figure 6 Western blot analysis of the reaction between the rOmp-G and the sera of survival eels challenged with three *Aeromonas* strains of B10, B27, and B33 The rOmp-G reacted with the sera of each survival eel after bacterial challenges. M, low molecular weight marker; 1–3, reactions between the rOmp-G and the sera of survival eel challenged with B10, B27, and B33 strain, respectively; 4, reaction between the rOmp-G and the serum of negative control eel. The arrows indicate the rOmp-G with a molecualr mass of 55.2 kDa.

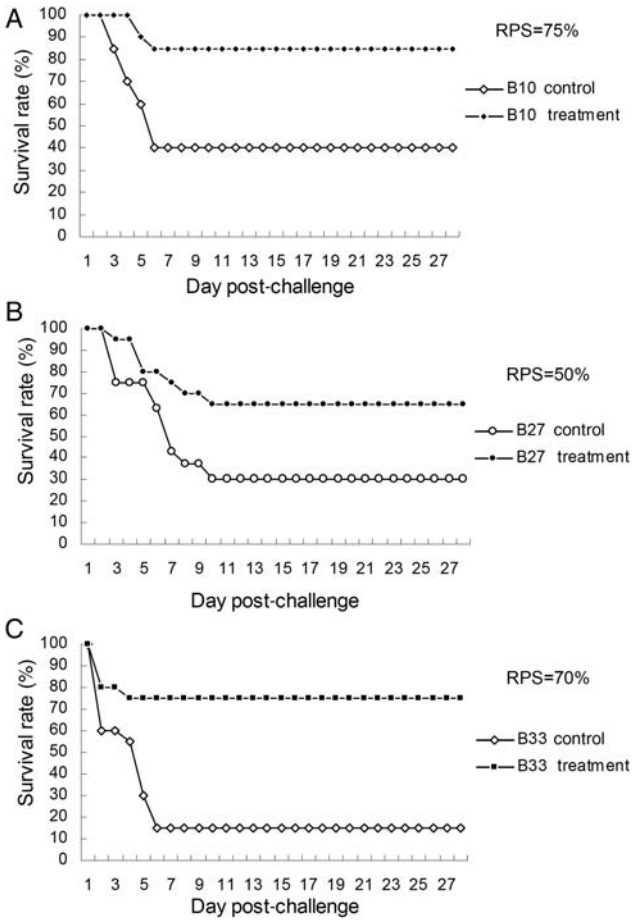

Figure 7 Survival rates of eels after challenged with three *Aeromonas* strains The relative survival rates were 75%, 50%, and 70% for B10, B27, and B33, respectively. Relative survival rate was calculated as follows: RPS = (1 − mortality in immunized fish%/mortality in control fish %) × 100.

not been done [19]. In this study, we constituted a recombinant OMP (rOmp-G), which provided protective immunogenicity for European eels to defend themselves against one *A. sobria* and two different serotypes of *A. hydrophila* strains at the same time [**Fig. 7(A–C)**]. It is the first report that a successful vaccination in eels can simultaneously provide protectiveness against three different *Aeromonas* pathogens by a partial OMP, and this is thought to be more efficient and feasible.

In the conventional approach to find the conserved OMPs in different bacterial strains, SDS-PAGE, 2D-PAGE, western blot, or other experimental technologies are adopted [15–17,20–21,24–28], but normally they are time-consuming, labor-intensive and expensive for the OMPs extraction and purification. In this study, we chose a convenient way to screen the conserved OMPs antigens from different bacterial pathogens by searching and blasting the NCBI database. With the rapid development of genomics and proteomics, many antigen sequences have been found and submitted to the database, which make it easy to search and compare the conserved antigens from different pathogens. Based on this idea, we searched and analyzed about 1000 reported OMP nucleotide sequences of fish pathogens in the NCBI database, and fortunately got 7 OMP nucleotide sequences having the homologies of more than 97%. Analyzed with DNAMAN 5.0 software, a high conserved region of 747 bp in these seven OMPs sequence was chosen as the target-conserved fragment, and the deduced protein of this 747-bp fragment was predicted to have good immunogenicity. Because this was our first time to search conserved OMPs from the reported OMPs in the database, we only chose those OMPs with high homologies of more than 97%. This made all of these seven OMPs found in recent database belonging to the same *Aeromonas* genus, and also restricted the disease prevention scope to some degrees. Thus, if a little lower homology (from 75 to 90%) or a shorter conserved fragment (<747 bp) was chosen, the shorter part of Omp-G reported in this study or some other conserved OMP fragments from different genus bacteria might be found. And, with the database enlargement, we believe that more and more conserved OMP nucleotide sequences belonging to the same or different genus would be revealed, which could help to enlarge the disease prevention scopes to different genus. In the same way, other antigens such as flagellum proteins, hemolysin, or other kinds of OMPs, which are highly conserved in different pathogenic bacterial strains or species, could also be found by searching and blasting from the database.

According to the conserved OMP fragment in the database, four pairs of specific primers were designed to amplify the conserved OMP from 20 pathogenic bacterial strains by PCR, but the right size of PCR products were only gained with one of the four pairs of primers in the PCR amplification (data not shown). Although the specific primers were designed according to the conserved OMPs sequences of *A. hydrophila* strains, two different serotype *A. hydrophila* strains and one *A. sobria* strain were positive to amplify the conserved OMP fragment (named as Omp-G). These three *Aeromonas* pathogens were isolated from different species of eels in different areas of China at different times [29]. This OMP fragment region was found to be conserved in 15 strains of different known *Aeromonas* pathogens with the identity of more than 77% in the NCBI database (**Table 1**). The designed primers used in this study are specific and the annealing temperature used in the PCR program was high (62°C). More positive PCR products might be gained if the PCR was performed at lower annealing temperature. In previous work, a similar conserved OMP nucleotide (ompTS) was also amplified from two *A. sorbia* strains: *A. hydrophila* strain and *A. caviae* strain [33], which further confirmed the method introduced in this study to find vaccine candidates was efficient and feasible. In the present research, we only chose 20 pathogenic bacterial strains to amplify the conserved Omp-G; therefore, further studies are necessary to find out whether this conserved Omp-G does exist in other pathogenic bacteria species.

The prediction of Hidden Markov model method showed that the deduced Omp-G protein was classified into porin protein family, which indicated that it had good immunogenicity and was a suitable candidate for developing vaccine against bacterial infections [16]. In this study, the specific sera against this rOmp-G by immunizing the rabbits and eels were detected by ELISA. The results showed that the rOmp-G had good immunogenicity for eliciting good humoral immune responses both in rabbits (**Table 2**) and in eels (**Fig. 5**), which was in accordance with other studies [33,34]. All the results showed that the rGST-His tag did not affect the immunogenicity and specificity of the rOmp-G. At the same time, the results also showed that the rOmp-G was recognized by the rabbit anti-sera (**Table 2**), as well as by the eel anti-sera (**Fig. 6**), suggesting that the rOmp-G has contributed to some conserved epitopes of these three *Aeromonas* pathogens, and is involved in their immunogenicity [16,21–22,24,26]. In addition, the S-rOmp-G agglutinated with the whole cells of the three bacteria, suggesting that the specific antibody induced by the rOmp-G can recognize the conserved OMP epitopes on the cell surface of the three *Aeromonas* pathogens and may provide cross-protective immunogenicity [21]. The BLAST results showed that the Omp-G gene or protein was well conserved among different *Aeromonas* strains or species (**Table 1**), so if this conserved Omp-G is really distributed among other pathogens except for the three *Aeromonas* strains (B10, B27, and B33), the encoding proteins might be used to prevent more pathogenic bacterial diseases in fish cultivation.

In conclusion, the rOmp-G, as the potential vaccine candidate, has been shown to enhance the protective immunity in European eels with good immunogenicity, and may also be used to enlarge the prevention scope for bacterial infections to some degree. In order to enlarge the disease prevention scopes, further work should be done to search for other well-conserved antigens such as flagellum proteins, hemolysin, or other kinds of OMPs from *Vibrio*, *Edwardsiella* or different fish pathogens to explore efficient polyvalent vaccines.

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