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Identification of polyvalent protective immunogens from outer membrane proteins in *Vibrio parahaemolyticus* to protect fish against bacterial infection

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ABSTRACT

Vaccination is one of the most effective and economic way to prevent infectious diseases in aquaculture. The development of effective vaccines, however, is still limited, especially for polyvalent vaccines, which are against multiple species. With this regard, identification of polyvalent protective immunogens, serving as polyvalent vaccines, became a key step in vaccine development. In the current study, 17 outer membrane proteins from *Vibrio parahaemolyticus* were identified as immunogens. Further, four of the 17 proteins including VP2309, VP0887, VPA0548 and VP1019 were characterized as efficiently protective immunogens against *V. parahaemolyticus*' infection through passive and active immunizations in zebrafish. Importantly, these four proteins showed cross-protective capability against infections by *Aeromonas hydrophila* or/and *Pseudomonas fluorescens*, which shared similar epitopes with *V. parahaemolyticus* in homology of these proteins. Further investigation showed that the expression level of the four protective immunogens elevated in response to fish plasma in a dose-dependent manner. These results indicate that the four protective immunogens are polyvalent vaccine candidates in aquaculture.

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1. Introduction

Aquaculture is an important component of economic relevance worldwide [1]. The outbreaks of microbial diseases results in huge economic losses in this sector, which is considered the major problem associated with aquaculture, food safety and import and export economics [1,2]. Although the administration of antibiotics represents a simple and relative low-cost solution to bacterial infections, limited success has been achieved due to the emergence of multidrug-resistant bacteria, microorganism substitution, ecological and public health impacts [3,4]. Thus, effective vaccines are needed to manage bacterial infections for improving food quality and safety in aquaculture.

Vibrios are ubiquitous marine bacterial species found in a wide range of aquatic habitats. They are frequently encountered in marine aquaculture animals. Some species of *Vibrios* like *Vibrio parahaemolyticus* and *Vibrio alginolyticus* have long been considered as fish pathogens in marine environments and presents as an important limitation to aquaculture production [5–7]. Meanwhile, *Pseudomonas fluorescens* and *Aeromonas hydrophila* are also common pathogens to fish, causing huge economic loss in aquaculture [8,9]. Although administration of antibiotics is the routine way to manage bacterial pathogens, it results in the generation of resistant strains [10,11]. Thus, alternative strategies like vaccines, phage therapy, antibacterial peptides and fish warfare have been developed to reduce the risk of the development and spreading of microbial resistance in aquaculture [12–17]. Among these different strategies, vaccination is the most effective way to combat the infectious diseases. Thus, identification of protective immunogens is especially important for the development of effective vaccines [18–20]. Of notice, a variety of bacterial pathogens are present in aquaculture, raising the concern of efficiency of monovalent vaccines targeting one species. Although a mixture of monovalent vaccines can be applied, different vaccines together may lead to immune interference of each other. In this case, polyvalent protective immunogen is a more optimistic strategy to overcome those

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limitations, and present as an more efficient and economic way [12,18,19]. Thus, identification of polyvalent vaccine is important in aquaculture.

Recently, we have identified VP1061 and VP2850 from six outer membrane proteins of *V. parahaemolyticus* as polyvalent vaccines against *V. parahaemolyticus* and other two key aquaculture pathogens *Pseudomonas fluorescens* and *Aeromonas hydrophila* using our developed cross-immunoproteomics approach [12]. This motivates us to propose outer membrane proteins are a rich pool for polyvalent protective immunogens in Gram-negative pathogens. Thus, we expand our previous study and explore more polyvalent protective immunogens from outer membrane proteins of *V. parahaemolyticus*.

2. Material and methods

2.1. Bacterial strains, culture and fish feeding

The bacterial strains in this study were *Vibrio parahaemolyticus, Aeromonas hydrophila* and *Pseudomonas fluorescens*. All strains were from the collections in our laboratory. A single colony of each strain was propagated in Luriae Bertani (LB) medium at 30 °C for 16 h. The culture was diluted 1:100 into fresh LB medium and grown to an OD 600 nm of 1.0. Zebrafish (*Danio rerio*) (average weight 0.3 g) for animal research were purchased at Huadiwan in Guangzhou. Before experiment, the fish were acclimated in 25 L open-circuit water tanks with aeration for two weeks and were demonstrated to be free of *Vibrio* species through zebrafish homogenates being cultured in TCBS agar. The fish were fed on a balanced commercial diet (Hikari Tropical Fancy Guppy, Kyorin, Hyogo, Japan) containing 33% crude protein, 4% crude fat, 17% crude ash related to wet matter and 10% moisture, at a ratio of 3% of body weight per day. The fish were anesthetized by immersion in 100 ng/ml of tricaine methanesulphonate (MS-222, Sigma, USA) before intraperitoneal injections and were immersed in Tris-buffered MS-222 at 300 ng/ml for at least 10 min for euthanasia as described previously [21].

2.2. Gene cloning and recombinant protein expression

Seventeen genes encoding *V. parahaemolyticus* outer membrane proteins with an unknown function on immune protective ability were selected. According to the complete genome sequence of *V. parahaemolyticus* (NCBI accession No. NC_004603.1 and No. NC_004605.1), seventeen pairs of corresponding primers were designed as shown as in Table 1. Cloning and expression procedures were carried out using standard procedures as described previously [12]. In brief, PCR procedure was a predenature cycle of 94 °C for 4 min; 35 cycles of 94 °C for 1 min, 54–60 °C for 1 min, 72 °C for 30 s; and a final extension at 72 °C for 10 min. The amplified target genes were cloned into pET-32a or −28a expression vector (Novagen). The resulting recombinant plasmids were transferred into *Eschherichia coli* DH5a (Novagen), checked by double digestion (Table 1) and transferred into BL21 (Novagen) for expression. The bacteria harboring target gene were induced by 0.2 mM isopropyl-b-D-thiogalactopyranoside (IPTG). The resulting recombinant proteins were identified by SDS-PAGE and used for protein purification.

2.3. Purification of recombinant proteins and preparation of antiserum

Harvested bacteria were resuspended with 50 mM Tris-HCl, and disrupted by intermittent sonic oscillation for a total of 15 min at 9 s intervals on ice as described previously [18]. The soluble recombinant proteins were purified by affinity chromatography Ni^{2+}-nitriloaceate (QIAGEN), while the recombinant proteins in inclusion were washed by scrubbing solution I and II, dissolved by 8 M urea, purified by affinity chromatography Ni^{2+}-nitriloaceate and dialyzed with gradient concentration of urea solution. Purity and concentration of the purified recombinant proteins were determined by SDS-PAGE and Bradford method, respectively. The proteins were diluted with sterile Tris-HCl (50 mM) to final concentration of 1 mg/mL. The purified recombinant proteins were used to immunize New Zealand rabbits, one rabbit each immunogen. The rabbits were firstly injected by 500 μg/100 μL purified proteins with 100 μL of Freund's complete adjuvant. Two weeks later, boost immunization consisted of three injections of 250 μg/100 μL purified proteins with 100 μL of Freund's incomplete adjuvant at two weekly intervals. Sera were collected and stored −80 °C for use. The protocol was approved by the Committee on the Ethics of Animal Experiments of Sun Yat-sen University.

Titers of antisera were quantitatively determined by Western blotting. Western blotting was performed as previously described [22]. In brief, *V. parahaemolyticus* outer membrane proteins from the gels were transferred to nitrocellulose (NC) membranes for 1 h at 70 V in transfer buffer (48 mM Tris, 39 mM glycine and 20% methanol) at 4 °C. The membranes were blocked with 5% skim milk and then incubated with 1:250, 1:500, 1:3,000, 1:5,000, 1:15,000, 1:25,000 and 1:50,000 dilutions of antisera against these tested outer membrane proteins as the primary antibodies. Horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG was used as the secondary antibody. The highest dilution was determined the titers of these antisera. When the antisera with the titers were higher than 1:500, they were adjusted to 1:500 by PBS for use when substrate (dimethyla-minoazobenzene) was optimum colored in 10 s (approximately IOD = 10).

2.4. Investigation of immune protection by passive immunization

Passive immunization was performed as previously described with a few modifications [12]. Zebrafish were randomly divided into two controls and seventeen experimental groups, and were further acclimatized for 2 weeks. Antisera against the seventeen outer membrane proteins were adjusted to same titer 1:500, and then were separately injected into zebrafish, 2 μL each. Zebrafish were injected with 2 μL of antiserum against a fusion protein from PET-32 for control-1 and with 2 μL of PBS for control-2. Three hours later, the zebrafish were challenged with 2 μL of *V. parahaemolyticus* at a concentration of 2.46×10^5 CFU through intraperitoneal injection. Passive immune protective effect was assessed by relative percent survivals (RPS) of zebrafish at 15th days post-immunization.

For cross-protective immunization, zebrafish were randomly divided into a control groups and four experimental groups with forty fish for each group. The four experimental groups were injected with antisera, and the control group with antiserum to fusion protein. After 3 h, both of the control and experimental groups were divided to two subgroups and separately challenged with 2 μL of 3.2×10^5 CFU of *A. hydrophila* or 1.44×10^6 of *P. fluorescens* by intraperitoneal inoculation. Cross-protective effect was assessed by RPS of zebrafish at 15 days. The protocols above were approved by the Committee on the Ethics of Animal Experiments of Sun Yat-sen University.

2.5. Investigation of immune protection by active immunization

Investigation of active immunization was carried out as described previously with a few modification [12,23]. Zebrafish were randomly divided into groups, 20 fish each. Each fish was intraperitoneally injected with 1.5 μg recombinant proteins

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