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Expression and Immunogenicity Analysis of Two Iron-regulated Outer Membrane Proteins of *Vibrio parahaemolyticus*

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Abstract Genes of two iron-regulated outer membrane proteins of *Vibrio parahaemolyticus* zj2003, a pathogenic strain isolated from large yellow croaker (*Pseudosciaena crocea*), *psuA* and *pvuA*, were cloned and expressed as N-terminal His_6-tagged proteins in *Escherichia coli* $BL_{21}(DE_3)$. The recombinant fusion proteins were purified with nickel chelate affinity chromatography. To analyze the immunogenicity of the proteins, groups of large yellow croaker were immunized with the purified recombinant psuA, pvuA or both, by intraperitoneal injection. Antibody response was assessed by enzyme-linked immunosorbent assay. Titers to the recombinant proteins increased from $\log_2 3.25$ to $\log_2 9.80$, 4–8 weeks following immunization. The relative percent survival of the groups vaccinated with psuA, pvuA, or a combination of the two, reached 50%, 62.5% and 75%, respectively. Western blot analysis was carried out with the serum from unvaccinated survival fish after infection. Both recombinant proteins were detected, indicating that these two proteins of *V. parahaemolyticus* zj2003 were immunogenic and could produce synergistic effects during *in vivo* infection, and they might be considered as important components for developing an aquaculture vaccine against this pathogen.

Keywords *Vibrio parahaemolyticus*; iron-regulated outer membrane proteins; gene cloning; prokaryotic expression; immunogenicity

Iron is one of the essential nutrients required for most microorganisms. However, for pathogenic bacteria invading a vertebrate host, the availability of free iron is very limited because most is complexed to lactoferrin, transferrin, heme and hemoglobin. Bacteria have evolved a number of diverse mechanisms to use the host iron. The best-studied mechanism is mediated by the low molecular weight high affinity iron chelators, termed siderophores, and cell surface receptor proteins specific for iron siderophore complexes, referred to as iron-regulated outer membrane proteins (IROMPs). In Gram-negative bacteria, the iron-siderophore complexes are translocated into the cytoplasm by outer-membrane receptors coupled with ATP-binding cassette-type transporters [1,2]. Antibodies against such receptors might block the related iron uptake system and induce protective immunity in the hosts [3–6].

Vibrio parahaemolyticus is a universal marine pathogen that causes vibriosis of fishes, crustaceans and bivalve mollusks [7–10]. In the coastal provinces of eastern China, *V. parahaemolyticus* is one of the causative agents of vibriosis that endangered the aquaculture of large yellow croaker (*Pseudosciaena crocea*). To date the only effective therapy has been antibiotics. Vaccines have been successfully used to control vibriosis in aquaculture [11], and IROMPs have been discovered to be highly immunogenic and should be included in vaccine formula for several Gram-negative pathogens [12–17]. *V. parahaemolyticus* is capable of acquiring iron through the action of the native siderophore vibrioferrin [18] and of using heme compounds as sole iron sources [19]. Under iron-restricted conditions, the bacteria produced a siderophore, vibrioferrin, accom-

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panying expression of two outer membrane proteins of 78 kDa and 83 kDa, the heme receptor and ferric vibrioferrin receptor, respectively [19,20]. The gene encoding the 78 kDa protein was cloned and named *pvuA*, preceded by the gene *psuA*, encoding a receptor of a siderophore of unknown origin [20]. Both IROMPs are important for the bacteria, because mutation of either of the two genes damaged the growth of the bacteria to some extent [21].

In the present study, we cloned and expressed the two *IROMP* genes, *psuA* and *pvuA*, of *V. parahaemolyticus* zj2003, a pathogenic strain isolated from diseased large yellow croaker. His_6-tagged recombinant proteins were expressed and purified, and injected into the fish intraperitoneally. Antibody response was detected by indirect enzyme-linked immunosorbent assay (ELISA) and assessed further by Western blot analysis. The relative percent survival (RPS) was recorded after experimental challenge with live bacteria. The significance of the two IROMPs for vaccine development was evaluated.

Materials and Methods

Bacterial strain

V. parahaemolyticus zj2003 was isolated from diseased large yellow croaker in Xiangshan Bay (Xiangshan, China).

Cloning *IROMP* genes and constructing recombinant prokaryotic expression plasmid

Two *IROMP* genes of *V. parahaemolyticus* zj2003 with complete open reading frames, *psuA* and *pvuA*, were cloned based on the published sequence of *V. parahaemolyticus* RIMD210633 (GenBank accession No. BA000032) and the two sequences were submitted to GenBank with the accession Nos. DQ141607 and DQ141608, respectively (data not shown). Oligonucleotide primers P1–P4 with appropriate restriction sites at the 5′ terminal were designed to amplify mature peptide coding genes as follows: P1, 5′-CGGGATCCTCAGAAGAGACAAACTCAACCC-3′ and P2, 5′-ACGCTCGAGCTAGAACTGGTATCCGTAGCTA-3′ with *Bam*HI/*Xho*I restriction sites (underlined) for *psuA*; P3, 5′-TAGAATTCGCTCCTGCCGCAAAAAACGAAA-3′ and P4, 5′-CGGCTCGAGTTAAAACTGATAGTTCAGATCC-3′ with *Eco*RI/*Xho*I (underlined) for *pvuA*. Genomic DNA prepared from *V. parahaemolyticus* zj2003 was used as the template. Polymerase chain reaction products were digested with restriction enzymes and ligated to pET30a (+) (Novagen, San Diego, USA). The constructs were confirmed by DNA sequencing and the resulting recombinant plasmids pET30a-IROMP were transformed to *Escherichia coli* $BL_{21}(DE_3)$ (Novagen) for expression.

Expression of recombinant protein

One liter cultures of *E. coli* $BL_{21}(DE_3)$ transformed with pET30a-IROMP were grown in Luria Broth at 37 ºC from a 1:100 dilution of initial inoculum. It was induced with 1 mM isopropyl β-*D*-thiogalactopyranoside when absorbance (A_{600}) of the culture reached 0.4–0.6. The cell cultures continued to grow at 37 ºC for 4 h. As a control, strain $BL_{21}(DE_3)$ transformed with pET30a(+) was treated with the same procedure simultaneously. The bacteria culture (1 ml) was centrifuged at 6000 *g* for 15 min and the pellets were collected. The pellets were suspended in sample buffer solution [2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% 2-mercaptoethanol, 0.003% bromophenol blue, and 0.0625 M Tris (pH 6.8)] and boiled at 100 ºC for 5 min. Total proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) on 12% (*W*/*V*) gel and stained with Coomassie Brilliant Blue. Molecular mass of proteins was deduced with Bandscan software (Glyko, Novato, USA).

Purification of recombinant protein

The recombinant proteins were purified with an agarose resin covalently coupled to iminodiacetic acid that binds Ni^{2+} ions by three coordination sites (Ni-IDA) (Zhuoguan Biotechnology, Beijing, China) according to the manual. Briefly, the bacteria cells were resuspended with buffer A (50 mM phosphate, 200 mM NaCl, pH 7.4), and lysed by sonication on ice. After centrifugation at 12,000 *g* for 10 min at 4 ºC, the supernatant was passed through a 0.45 μm filter. Then the filtered recombinant fusion proteins were loaded on Ni-IDA agarose resin, washed with buffer A and buffer I (50 mM phosphate, 200 mM NaCl, and 50 mM imidazole, pH 7.8), eluted with buffer II (50 mM phosphate, 200 mM NaCl, and 300 mM imidazole, pH 7.8), and dialyzed against 10 mM PBS, pH 7.8 for 48 h. The purities of recombinant fusion proteins were determined by SDS-PAGE. Protein concentration was adjusted to 0.5 mg/ml and stored at –80 ºC.

Preparation of formalin killed cells (FKC) of *V. parahaemolyticus* zj2003

Bacteria *V. parahaemolyticus* zj2003 were cultured in Zobell2216E media at 28 ºC for 14–16 h, and the cells were harvested by centrifuging at 6000 *g* for 15 min. The pellets were washed three times with sterile saline, then formaldehyde was added to the suspension to a final concentration of 0.5% (*V*/*V*). The suspension was

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incubated at 28 ºC for 24 h and tested by adding dilution on thiosulfate citrate bile salts sucrose (TCBS) agar plates for complete inactivation. The cell density was adjusted to the concentration of 1×10^9 cells/ml. That is the FKC prepared for vaccination the fish.

Fish vaccination

Large yellow croakers with an average body weight of 150 g were randomly divided into five groups, with 40 fish in each group. Groups 1, 2, and 3 were intraperitoneally injected with 100 μg purified recombinant psuA, pvuA, or the two proteins combined, respectively; positive control group 4 and negative control group 5 were injected with 0.2 ml FKC and 0.2 ml sterile 0.01 M PBS (pH 7.4), respectively.

Experimental challenge and calculation of RPS

Four weeks post-vaccination, 10 fish in each group were experimentally challenged by intraperitoneal inoculation with 0.2 ml of 1×10^8 c.f.u./ml of *V. parahaemolyticus* zj2003 cell suspension. Bacterial suspension was prepared as follows: strain of *V. parahaemolyticus* zj2003 was grown overnight in Zobell2216E broth at 28 ºC, harvested by centrifugation at 4000 *g* for 15 min and washed with PBS three times. Bacteria were suspended in 0.01 M PBS (pH 7.4) and adjusted to the concentration of 1×10^8 c.f.u./ml. Cumulative mortality and clinical signs were recorded daily for two weeks post-challenge and dead fish were autopsied to determine the cause of death and for the presence of *V. parahaemolyticus* in the tissues by bacterial culture in TCBS agar. The RPS was calculated as shown in **Equation 1**:

$$\text{RPS}=1-\left(\frac{\text{\% mortality in vaccinated fish}}{\text{\% mortality in control fish}}\right)\times100\% \quad (1)$$

In a preliminary experiment, an infection test was carried out to determine the appropriate bacteria concentration for experimental challenge. Two weeks after intraperitoneal inoculation, the sera of survival fish were collected for Western blot analysis.

Analysis of antibody response

Before the fish were immunized, 10 fish were bled and the sera were collected as negative control. At 4–8 weeks post-vaccination, five fish from each group were assayed for antibody response against various antigens by ELISA. Blood was collected from the caudal vein with a sterile syringe. After coagulation, the blood was centrifuged and the serum was collected and stored at −80 ºC. In short, 96-well plates were separately coated with each antigen (recombinant 8 μg/ml psuA, 8 μg/ml pvuA, and the boiled bacteria suspension of *V. parahaemolyticus* zj2003, 1×10^9 c.f.u./ml). Two-fold serial dilutions of the sera with the first dilution 1:8 were added to the double wells of the plates. Antibodies binding to the antigens were detected using rabbit anti-large yellow croaker immunoglobulin (Ig) polyclonal antibody (1:500), followed by goat anti-rabbit IgG conjugated with horseradish peroxidase (Dingguo Biotechnology, Beijing, China) at 1:1000 dilution. The color was developed with O-phenylenediamine for 30 min and the reaction was stopped with 2.0 M H_2SO_4. The plates were read with a microplate reader (Thermo Labsystems, Helsinki, Finland) 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.

Western blot analysis of recombinant protein

Protein samples were electrophoresed on 12% SDS-PAGE gel, then electroblotted onto a 0.45 μm nitrocellulose membrane at a constant current of 300 mA at 4 ºC for 1.5 h. After blocked in TBS (150 mM NaCl, and 20 mM Tris-base, pH 7.4) with 5% (*W*/*V*) skimmed milk, the membrane was incubated with the serum of survival fish after infection (1:100 diluted in TBS) at 37 ºC for 1.5 h. After washing with TBS and 0.1% Tween-20, the membranes were incubated with the second antibody, rabbit anti-large yellow croaker Ig polyclonal antibody (1:500) at 37 ºC for 1.5 h. After washing three times, goat anti-rabbit IgG conjugated with horseradish peroxidase was added and incubated for 1 h at room temperature. The membranes were visualized with 3,3′,5,5′-tetramethylbenzidine membrane substrate (Amresco, Solon, USA). The negative control sera were treated with the same protocol.

Statistical analysis

To evaluate the significance of the differences in immune response and protection between vaccinated and unvaccinated fish, three statistical analyses were carried out: the paired Student's *t*-test and Duncan's multiple range test to compare antibody titers; and the χ^2-test to compare mortality values. Values were considered significantly different at $P<0.05$.

Results

Polymerase chain reaction amplification of *IROMP* genes

Two *IROMP* genes without signal coding nucleotides

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were successfully amplified and appropriate restriction sites were introduced at the 5′ terminal of the products (**Fig. 1**). Sequencing and BLASTN (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi) results showed that both of the genes shared a high identity of 98% with the standard *V. parahaemolyticus* strain RIMD 2210633.

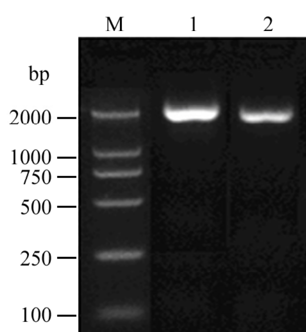

Fig. 1 Polymerase chain reaction amplification of two iron-regulated outer membrane protein genes of *Vibrio parahaemolyticus* zj2003
The mature peptide coding the genes was amplified, and appropriate restriction sites were introduced at the 5′ terminal of the products and visualized on 1% agarose gel. M, DNA molecular marker; 1, *pvuA*, 2028 bp; 2, *psuA*, 1962 bp.

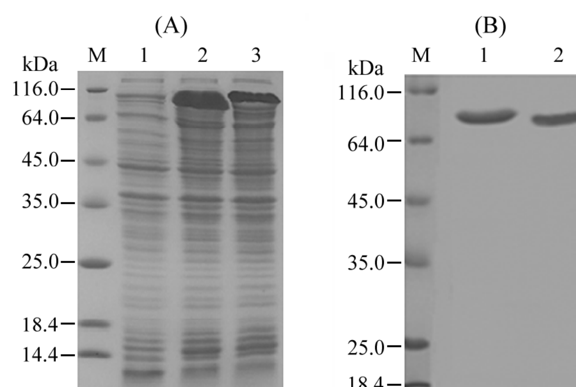

Fig. 2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) profiles of the total proteins of *Escherichia coli* BL21(DE3) transformed with pET30a-IROMP and purified recombinant proteins
(A) Protein expression was induced with 1 mM isopropyl β-*D*-thiogalactopyranoside at a cell density (A_{600}) of 0.4–0.6 and the culture was continued for 4 h. Total proteins of the bacteria were separated with 12% SDS-PAGE gel and stained with Coomassie Brilliant Blue. The molecular mass of the recombinant fusion proteins revealed by SDS-PAGE was analyzed by Bandscan software). M, protein marker; 1, total proteins of *E. coli* BL21(DE3) transferred with pET30a; 2, total proteins of *E. coli* BL21(DE3) transferred with pET30a-psuA; 3, total proteins of *E. coli* BL21(DE3) transferred with pET30a-pvuA. (B) Recombinant proteins were purified with His_6-tag affinity chromatography and separated on 12% SDS-PAGE gel. M, protein marker; 1, purified recombinant pvuA; 2, purified recombinant psuA.

Analysis of recombinant fusion proteins

The recombinant proteins were over-produced [**Fig. 2 (A)**]. The molecular masses revealed by SDS-PAGE were approximately 80 kDa and 78 kDa, in agreement with the theoretical values (80.77 kDa and 77.94 kDa). The recombinant proteins were produced as insoluable inclusion bodies (data not shown). After His_6-tag affinity chromatography, the expressed proteins were purified to single band, as confirmed by SDS-PAGE, and the purity was above 95% [**Fig. 2(B)**].

Protection of large yellow croaker from *V. parahaemolyticus* challenge

The vaccinated groups were assessed for protection against live *V. parahaemolyticus* challenge (**Table 1**). The cumulative percent mortality in the FKC and protein combination groups was significantly lower than that of the control (80%), being 10% and 20%, respectively. The cumulative percent mortality of pvuA or psuA group was a little higher, reaching 30% and 40%, respectively. When pvuA or psuA was used for vaccination, a middle level of protection was observed (RPS reached 62.5% and 50%, respectively). When the protein combination was used for vaccination, a higher protective effect was achieved, and RPS reached 75%. The group vaccinated with FKC had the highest RPS of 87.5%. Symptoms of congestion and ulcer were observed in diseased fish, and the inoculated bacteria *V. parahaemolyticus* was isolated from the kidney or liver of dying individuals.

Table 1 Cumulative percent mortality and relative percent survival (RPS) of vaccinated large yellow croaker (*Pseudosciaena crocea*), following intraperitoneal challenge with *Vibrio parahaemolyticus* zj2003

Vaccinated antigen	Cumulative percent mortality (dead fish/challenged fish)	RPS
PBS	80 (8/10)	—
FKC	10 (1/10)*	87.5%
psuA and pvuA	20 (2/10)*	75.0%
pvuA	30 (3/10)	62.5%
psuA	40 (4/10)	50.0%

Cumulative percent mortality and RPS of large yellow croaker vaccinated with various antigens or phosphate-buffered saline (PBS; negative control) following live bacteria challenge. Mortality data were collected daily for 14 d following challenge treatment. *$P<0.05$ versus control group, χ^2-test. FKC, formalin killed cells.

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Antibody response to immunization with recombinant proteins

Antibody titers of vaccinated groups specific for three different antigens are graphically represented in **Fig. 3**. When the recombinant protein psuA or pvuA was used as the ELISA antigen, a significantly high level of specific antibody titers was found in groups immunized with the protein or the protein combination; no significant antibody response was revealed in other treated groups [**Fig. 3(B, C)**]. The titers to recombinant pvuA increased during 4–7 weeks post-immunization, and declined a little in the following week, varying from $\log_2$ 6.0 to $\log_2$ 8.2 [**Fig. 3 (C)**]; titers to recombinant psuA continuously increased from $\log_2$ 3.25 to $\log_2$ 5.4 in the same time period [**Fig. 3 (B)**]. In the protein combination immunized group, titers to psuA and pvuA increased from $\log_2$ 2.8 to $\log_2$ 4.6 and $\log_2$ 5.0 to $\log_2$ 7.0, respectively, not significantly lower than the psuA alone or pvuA alone treatment groups. When the boiled whole bacteria was used as the capture antigen, only the FKC vaccinated group showed significantly high titers, with the peak of $\log_2$ 12.75 at week 4, but declined in the following weeks; other groups showed no significant differences from the control [**Fig. 3(A)**]. At the same time, in the PBS-injected control group, antibody titers to all three antigens were not over $\log_2$ 2.0.

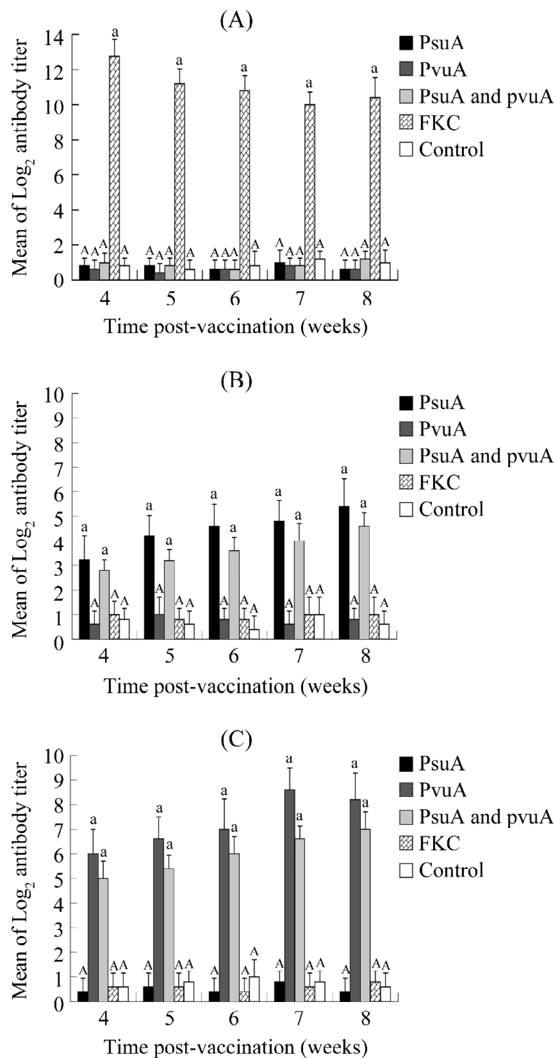

Fig. 3 Antibody titers of vaccinated large yellow croaker during weeks 4–8 post-vaccination

Sera collected at weeks 4, 5, 6, 7, and 8 post-vaccination were assayed by enzyme-linked immunosorbent assay for the presence of antibodies to immunized antigen. Serum collected before injection was used as the negative control. Antigens included boiled whole bacteria of *V. parahaemolyticus* zj2003 (A), recombinant psuA (B) and recombinant pvuA (C). Results are shown as $\log_2$ antibody titer. Each column represents the mean of $\log_2$ antibody titers with standard deviation bar. n=5. Letter "A" indicates no significant differences between the treated group and the control group; while letter "a" means significant difference between the groups. Differing letters indicate significant differences between treatments (paired Student's t-test and Duncan's multiple range test, P<0.05). FKC, formalin killed cells.

Western blot analysis of recombinant proteins with serum from unvaccinated survival fish after experimental infection

The immunogenicity of the recombinant proteins was further assessed using Western blot analysis with the serum of unvaccinated survival fish after experimental infection (**Fig. 4**). Both the recombinant psuA and pvuA

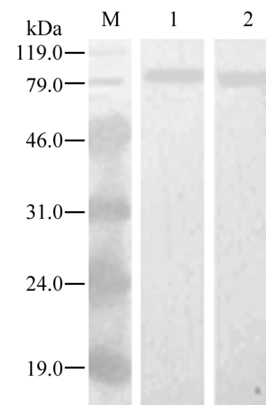

Fig. 4 Western blot analysis of recombinant proteins pvuA and psuA with serum of unvaccinated survival fish (large yellow croaker) after experimental infection with *Vibrio parahaemolyticus* zj2003

After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, purified recombinant proteins were transferred to nitrocellulose membranes. The blot was developed with the serum of unvaccinated survival fish after experimental infection. M, prestained molecular marker; 1, recombinant pvuA; 2, recombinant psuA.

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proteins were recognized by the serum. The negative control sera did not react with any of the recombinant proteins (data not shown).

Discussion

Previous papers have reported that pvuA, the siderophore vibrioferrin receptor, appeared to be conserved in *V. parahaemolyticus* strains of various origins [20,21]. In our study, we cloned the genes of *psuA* and *pvuA* from a pathogenic strain, *V. parahaemolyticus* zj2003, isolated from diseased large yellow croaker in a coastal area of eastern China. Sequence alignments revealed high identity (98%) shared with the published data, which also showed the conservation of these proteins.

IROMPs have been attractive candidates for vaccines because they are exposed to the surface of the outer membrane, and they are antigenic and might induce antibodies that block essential iron uptake of the bacteria. Protective immunity induced by IROMPs has been shown in many other Gram-negative bacteria, such as *E. coli*, *Pasteurella multocida*, *Salmonella typhi*, *Neisseria meningitidis* and *Aeromonas salmonicida* [12–17]. In the present study, we have observed the immunogenicity of the two IROMPs, psuA and pvuA of *V. parahaemolyticus*. Vaccination with each of the recombinant proteins elicited significant antibody response in the fish and conferred moderate protection against artificial challenge. When the combination of the proteins was used, significant levels of antibody titers to each of the proteins was produced, and a higher RPS was achieved. Moreover, fish convalescent sera recognized both of the recombinant proteins. It was indicated that the two recombinant proteins are immunogenic and, when given together, a synergistic effect in protective immunity was produced. The native proteins were expressed during *in vivo* infection. The results suggested that both of the proteins were involved in protective immunity against this pathogen. *V. parahaemolyticus* has evolved several iron uptake systems. We know that pvuA acts as the ferric vibrioferrin receptor, psuA is a receptor of siderophore of unknown origin, and the two genes are organized in an operon structure. Both of the proteins are essential components of iron uptake systems of the bacteria [21]. In *E. coli*, the synergistic effects of antibody against ferric citrate receptor and ferric enterobactin receptor on reduction of *in vitro* iron uptake were already reported [6]. In our study, antibody against the two IROMPs might block the ferric vibrioferrin-mediated and other iron uptake system *in vivo* and affect the survival and proliferation of the pathogen. When both of the systems are blocked, the growth of the bacteria would be severely damaged, which might account for the higher protection when the proteins were given in combination.

We have observed two immunogenic IROMPs of *V. parahaemolyticus* zj2003, and the two proteins produced synergistic effects in protective immunity. It is noteworthy that in our study, formalin killed whole cells bacterin acted better than the subunit vaccine of recombinant IROMPs, however, the efficacy of such bacterin would be doubtful if the serotype of the pathogen changed. As for the serotypes of *V. parahaemolyticus*, 11 O-antigens and over 70 K-antigen heterogeneities are present in clinical and environmental isolates [22,23], making it rather difficult to develop an efficacious vaccine for wide use. IROMPs are critical to the survival of the organisms *in vivo*, and become major outer membrane proteins under iron-restricted conditions. It seems pvuA and psuA of *V. parahaemolyticus* are genetically conserved among a variety of serotypes [21], and antibodies reactive with IROMPs might provide some cross-serotype protection. The present study has shown the immunogenicity of the two IROMPs of *V. parahaemolyticus*, which might be considered important vaccine components. At present, commercially available bacterins are usually prepared with enriched culture media containing a high level of iron, so it is unlikely that such vaccines would contain immunogenic IROMPs. However, these proteins could be bulk produced with engineering bacteria and be added to the bacterin. When considering both the efficiency and economy of the vaccine, this might be a solution for the serotype variety of this pathogen. Further study is needed to verify the molecular conservation of these IROMPs in various serotypes of the bacteria.

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