Fish and Shellfish Immunology 107 (2020) 64–72

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Recombinant *Vibrio parahaemolyticus* ghosts protect zebrafish against infection by *Vibrio* species

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ARTICLE INFO

Keywords:
Vibrio parahaemolyticus
Bacterial ghosts
Polyvalent vaccine
Immunoprotection

ABSTRACT

Aquatic animals are frequently threated by bacterial pathogens. The most economic and efficient protection against bacterial infection are through vaccine immunization. The various serotypes of the pathogens, such as Vibrios, hurdle the development of the vaccines, especially polyvalent vaccines. Here, we demonstrate that recombinant bacterial ghost is a good candidate for multivalent vaccine. By expressing PhiX174 gene E alone or co-expressing the gene E with two genes encoding outer membrane proteins (VP1667 and VP2369) in *V. parahaemolyticus*, we generated the recombinant *V. parahaemolyticus* ghosts VPG and rVPGs respectively. Fish immunized with either VPG or rVPG showed increased survival against the infection by either *V. parahaemolyticus* or *V. alginolyticus*, with a better protective effect by immunization with rVPG. Our furthermore studies show that rVPG stimulates stronger innate immune responses by increasing the expression of *tnfα, il1β, il6, il8* and *il10* as well as that of *c3b*, *lyz*, and *tlr5,* the key players linking the innate and adaptive immune responses upon microbial stimulation. In summary, VPG and rVPG can protect zebrafish against the infection from at least two *Vibrio* species, suggesting its potential value for further aquaculture vaccines development.

1. Introduction

The extracellular pathogenic Vibrio spp., including *Vibrio parahaemolyticus* [1–3], *Vibrio alginolyticus* [4,5] and *Vibrio anguillarum* [6,7], cause Vibriosis in aquatic animals [8] and could result in massive economic losses in aquaculture industry. Among these pathogens, *V. parahaemolyticus* causes acute hepatopancreas necrosis disease (AHPND) in shrimp, and it is also one of the major pathogens for cultured mud crabs [9,10]. The most adopted approach to control the infection by Vibrio species in aquaculture is to use antibiotics for treatment because of their high efficacies and low costs. However, the overuse and misuse of antibiotics in the aquatic environment often lead to the emergence of resistant bacteria, a major concern in fish and shellfish farming and to human health [11–14]. A variety of strategies, including the treatment of fish with vaccines, antibacterial peptides, antibiotic adjuvants, phage, metabolites, have been adopted to prevent or control the infectious diseases caused by bacterial pathogens in fish farming [15–21]. Among them, vaccination that can enhance host immunity has been demonstrated to be the most economic, efficient and environment-friendly strategy in protecting fish from bacterial pathogens [22,23]. Currently, the available vaccines are usually serotype- or species-specific, which makes their roles in preventing infections limited [24]. It is thus important to enhance the protective efficiency of vaccines against infectious diseases. Among various potential candidates, bacterial ghost (BG) has been proposed as a candidate for a multivalent vaccine [25–29].

BGs are empty non-living cell envelopes derived from Gram-negative bacteria. They preserve entire cell surface structures of the bacteria, including lipids, outer membrane proteins, lipopolysaccharides (LPS), adhesins and the peptidoglycan layer [27], and the foreign antigens can be expressed either on the surface or in the periplasmic space of BGs [30,31]. These remarkable properties allow BGs to represent a potential platform for vaccine development and antigen delivery system for both humans and animals. The common method to generate BGs is to express

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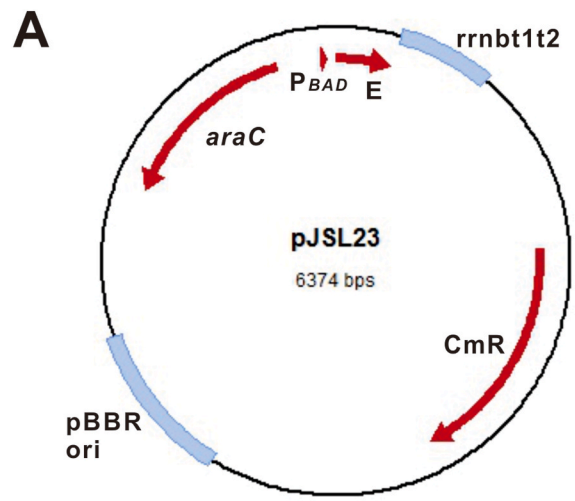
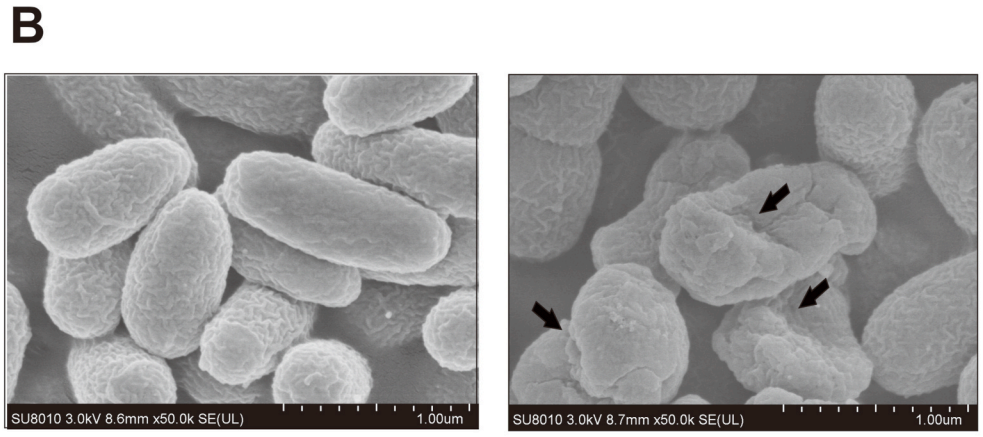
https://doi.org/10.1016/j.fsi.2020.10.009
Received 9 May 2020; Received in revised form 7 August 2020; Accepted 6 October 2020
Available online 7 October 2020





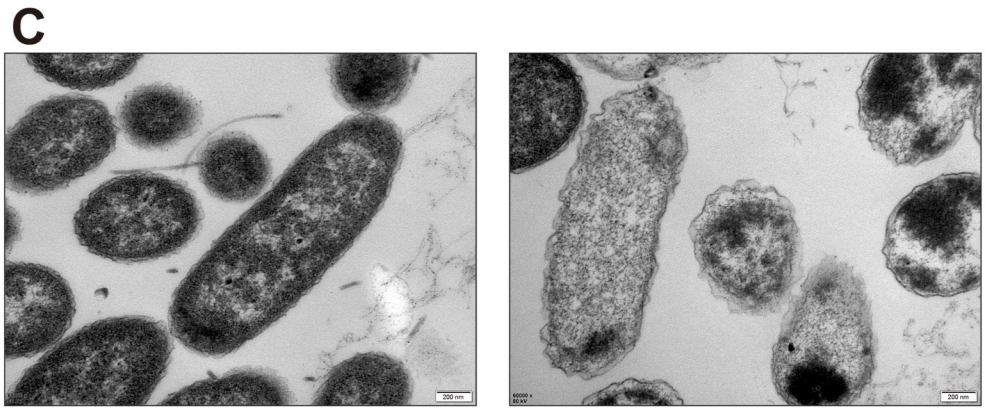


Fig. 1. Components of the ghost plasmid pJSL23 (A) and characterization of *V. parahaemolyticus* ghost cells by scanning electron microscopy (B) and transmission electron microscopy (C). (A) The *V. parahaemolyticus-E. coli* shuttle plasmid with pBBR ori carrying inducible expression system of the lysis genes are regulated by the arabinose operon. (B) SEM of the morphology of intact *V. parahaemolyticus* cells (left) and *V. parahaemolyticus* ghost cells (right). The transmembrane tunnel structure in VPGs is indicated by arrows. (C) TEM of intact *V. parahaemolyticus* cells (left) and *V. parahaemolyticus* ghost cells (right).

the gene E of bacteriophage PhiX174 in the target bacteria [32,33]. The expressed Phi X174 protein E oligomerizes into a transmembrane tunnel on the bacterial cell surfaces and results in the expulsion of cytoplasmic contents due to the high osmotic pressure inside bacterial cells, generating the empty cell envelopes of BGs [28,34]. BGs have been evaluated as candidate vaccines for immune protection against pathogens or viruses in various animal models [26,35]. The immunization of mice with *Salmonella typhi* Ty21a bacterial ghosts that carry various antigens of human immunodeficiency virus (HIV) led to stronger humoral responses to both the BG components as well as to the viral target proteins [36]. *Vibrio cholerae* ghosts (VCGs) induced antibodies showed vibriocidal activity and provided protection from homologous and heterologous bacterial challenges in animal models [37,38].

We recently showed that VCGs could act as an effective adjuvant to enhance the immune protective effect of classic swine fever live vaccine in rabbit [39]. A study by Seryun et al. showed that tilapia was protected from edwardsiellosis by vaccination with *Edwardsiella tarda* ghosts [40]. In this study, *V. parahaemolyticus* ghosts (VPGs) were generated by the expression of the cloned gene E of bacteriophage PhiX174 under the control of araBAD promoter. Zebrafish immunized by VPGs showed higher survival after being challenged by *V. parahaemolyticus* or *V. alginolyticus*. In addition, when two membrane proteins, VP1667 and VP2369, were co-expressed with the gene E in *V. parahaemolyticus*, the produced recombinant *V. parahaemolyticus* ghosts (rVPGs) demonstrated

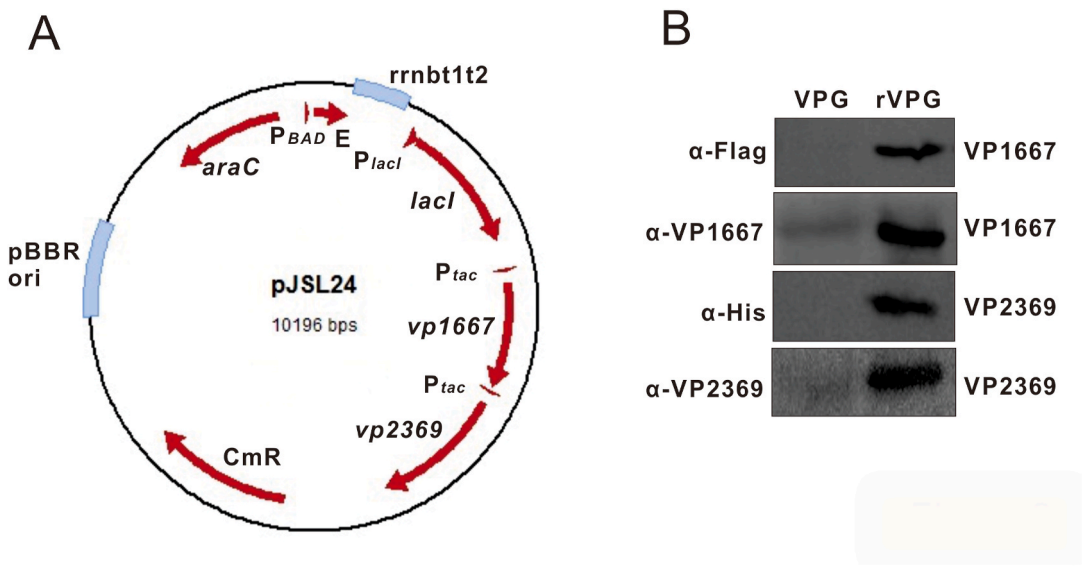


Fig. 2. VP1667-cFLAG and VP2369-cHis$_6$ are overexpressed in recombinant *V. parahaemolyticus* ghost cells. (A) Components of the ghost plasmid pJSL24 containing the arabinose inducible expression system of the lysis genes and the lactose inducible expression system of *vp1667* and *vp2369* genes. (B) 10 μg of membrane protein from VPGs or rVPGs was separated by SDS-PAGE. VP1667 and VP2369 were detected by Western blotting using relevant antibody. Blot shown is representative of at least three separate experiments.

Table 1
Primers of QRT-PCR for innate immunity genes.

Gene	Primer sequence(5′ to -3′)		Length
TNF-α	Sense	ATAAGACCCAGGGCAATCAAC	21
	Antisense	CAGAGTTGTATCCACCTGTTAAATG	25
IL-1β	Sense	TGGACTTCGCAGCACAAAATG	21
	Antisense	GTTCACTTCACGCTCTTGGATG	21
IL-6	Sense	ATCCGCTCAGAAAACAGTGCT	21
	Antisense	GTCGCCAAGGAGACTCTTTAC	21
IL-8	Sense	CACGCTGTCGCTGCATTG	18
	Antisense	GTCATCAAGGTGGCAATGATCTC	24
IL-10	Sense	CTCTGCTCACGCTTCTTC	18
	Antisense	TCATCGTTGGACTCATAAAAC	21
C3b	Sense	TGTGACCCGCTGTATGTTCT	20
	Antisense	TTGGCTGGGAAGTTCTTCAC	20
Lysozyme	Sense	GATTTGAGGGATTCTCCATTGG	21
	Antisense	CCGTAGTCCTTCCCCGTATCA	21
TLR5	Sense	GAAACATTCACCCTGGCACA	20
	Antisense	CTACAACAAGCACCACCAGAATG	23

Table 2
Cross-active immunoprotection of VPGs in the zebrafish model.

VPGs	Bacterium	Nos	Remain[a]	ADR (%)[a]	RPS (%)[a]
10^6-1st	*V. parahaemolyticus*	20	17	15	70[b]
	V. alginolyticus	20	18	10	80[b]
10^7-1st	*V. parahaemolyticus*	20	18	10	80[b]
	V. alginolyticus	20	17	15	70[b]
Control-1st	*V. parahaemolyticus*	20	10	50	–
	V. alginolyticus	20	10	50	–
10^6-2nd	*V. parahaemolyticus*	20	16.5	17.5	65[b]
	V. alginolyticus	20	19	5	90[b]
10^7-2nd	*V. parahaemolyticus*	20	17	15	70[b]
	V. alginolyticus	20	19.5	2.5	95[b]
Control-2nd	*V. parahaemolyticus*	20	10	50	–
	V. alginolyticus	20	10	50	–

[a] Average of two biological repeats. Control, 1 × PBS with the same injection volume as that of VPGs; ADR, accumulating death rates; RPS, relative percent survival; Relative percent survival was calculated as RPS = 1- (%mortality of vaccinated group/% mortality of control group) × 100.
[b] $P < 0.05$.

Table 3
Cross-active immunoprotection of rVPGs in the zebrafish model.

rVPGs	Bacterium	Nos	Remain[a]	ADR (%)[a]	RPS (%)[a]
10^6	*V. parahaemolyticus*	20	18	10	82[b]
	V. alginolyticus	20	18	10	80[b]
10^7	*V. parahaemolyticus*	20	19	5	91[b]
	V. alginolyticus	20	18	10	80[b]
Control	*V. parahaemolyticus*	20	9	55	–
	V. alginolyticus	20	10	50	–

[a] Average of two biological repeats. Control, 1 × PBS with the same injection volume as that of VPGs; ADR, accumulating death rates; RPS, relative percent survival; Relative percent survival was calculated as RPS = 1- (%mortality of vaccinated group/% mortality of control group) × 100.
[b] $P < 0.01$.

enhanced protective capability in zebrafish against the infection by either *V. parahaemolyticus* or *V. alginolyticus*. Therefor our rVPGs can effectively stimulate the innate immunity response in zebrafish and protect them against infection by *Vibrio* species.

2. Materials and methods

2.1. Bacterial strains, culture and zebrafish feeding

The bacterial strains, *V. parahaemolyticus* [41] and *V. alginolyticus* [42], are collections from our lab. *Escherichia coli* strains DH5α, BL21, and CC118λpir were used for general manipulation of plasmids, prokaryotic expression of proteins, and mobilization of plasmids into *V. parahaemolyticus*, respectively. The bacterial strains were grown at 37 °C in Luria-Bertani (LB) broth with 1% NaCl [43] (for *E. coli*) or with 3% NaCl (for *V. parahaemolyticus* and *V. alginolyticus*) containing appropriate antibiotics. Zebrafish (*Danio rerio*) (average weight 0.3 g) were purchased from a commercial supplier. Before experiment, these animals were acclimated in 25 L open-circuit water tanks with aeration for two weeks and were validated to be free of Vibrio species through zebrafish homogenates being cultured in TCBS agar. Fish was 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

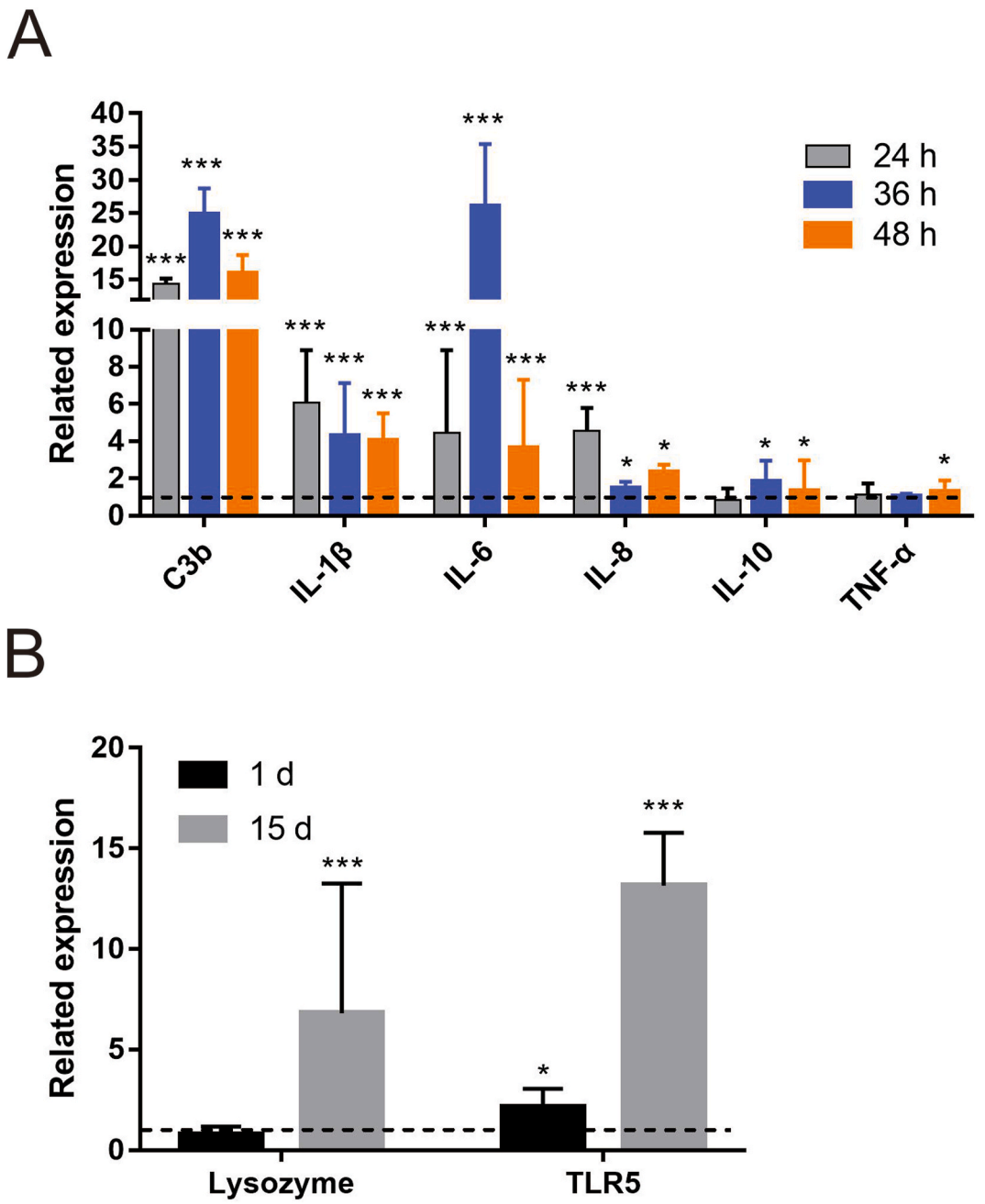


Fig. 3. QRT-PCR for expression of innate immune response in response to recombinant *V. parahaemolyticus* ghost cells. Zebrafish were immunized with rVPGs as tested groups and 1 × PBS as control respectively. Spleens were collected for determining expression of the innate immune response with QRT-PCR in different indicated time points, 24–48 h (A) or 1 and 15 days (B) after injection of rVPGs. Results above the dash line represent increased expression level compared with the control group. Data are mean and SD of three independent experiments. Statistical analysis was calculated by one-way ANOVA. n = 3. $^{*}P < 0.05$, $^{***}P < 0.001$.

per day. Fish was 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. All animal experiments were carried out in strict accordance with the animal protocols that were approved by the Institutional Animal Care and Use Committee of Zhejiang A&F University (Permit Number: ZJAFU/IACUC_2011-10-25-02).

2.2. Gene cloning and recombinant protein expression

V. parahaemolyticus VP1667 and VP2369 genes were amplified from the genomic DNA which was purified by QIAamp DNA Mini Kit (Qiagen) from bacterial culture. The PCR products without the signal peptide sequence were inserted into a modified pET-28a (Novagen, Inc.) vector encoding an N-terminal His_6-tag. *E. coli* BL21(DE3)/pLysS [44] cells were transformed with the plasmid containing the target gene and transformed cells were used for protein expression by autoinduction [45]. Proteins were expressed and purified on nickel columns according to the manufacturer's instructions (Invitrogen).

2.3. Antibody preparation

Ten milligrams of each protein, VP1667 and VP2369 purified as described above were used as antigens, and then sent to GenScript Inc. for polyclonal antibodies preparation by immunizing rabbits. Antibody expression or presence was detected by Western blotting of each specific

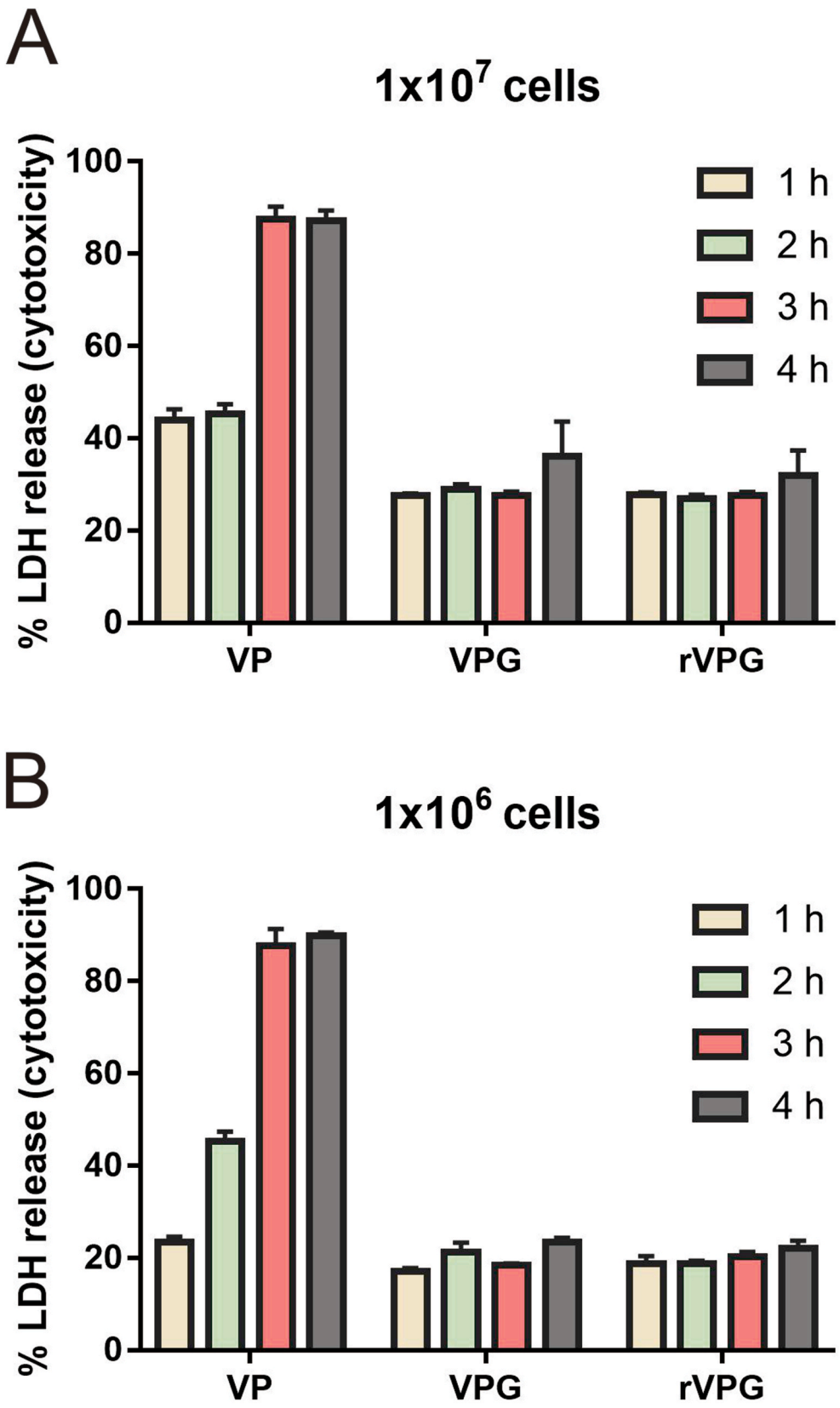


Fig. 4. Cytotoxicity assay of 1×10^7 (A) or 1×10^6 (B) of intact *V. parahaemolyticus* wild type cells, *V. parahaemolyticus* ghost cells and recombinant *V. parahaemolyticus* ghost cells against HeLa cells by detecting the release of LDH into the medium at each indicated time. Parameters reported include the mean ± SD across three replicates.

purified protein a week after the fourth immunization.

2.4. Detection of recombinant VP1677 and VP2369 by Western blotting

V. parahaemolyticus strains expressing or not VP1667 and VP2369 under the control of an isopropyl-β-D-thiogalactopyranoside (IPTG) inducible promoter were cultured at 37 °C with 200 rpm shaking in LB-NaCl medium until $OD_{600} \approx 0.5$. 0.5 mM of IPTG was added and bacteria were cultured in the same conditions for another 8 h. Membranes were prepared according to Kadokura and Beckwith [46] and 1 mg of the sample was separated by SDS-PAGE. VP1667 and VP2369 was detected by the Western blot using antiserum specific for either VP1667 or VP2369. Blot shown in Fig. 2 is representative of at least three separate experiments.

2.5. V. parahaemolyticus ghosts preparation

Recombinant VPG were produced by gene *E*-mediated lysis as described in Ref. [38]. *V. parahaemolyticus* harboring the lysis plasmid pJSL1 which could express the lysis *E* gene under the control of an arabinose inducible promoter was cultured at 37 °C with 200 rpm shaking in LB-NaCl medium until $OD_{600} \approx 0.5$. 0.2% of arabinose was added and bacteria were cultured in the same conditions for another 9 h. At the end of lysis, cultures were harvested by centrifugation, and washed with phosphate buffered saline (PBS). Harvested ghosts were resuspended in PBS and then lyophilized. The efficiency of E-mediated killing of *Vibrios* was estimated by plating samples of appropriate dilutions of freshly harvested and lyophilized VPG on LB-NaCl agar with and without antibiotics. Results indicated a 99.99% killing efficiency; no colony forming units were found on plates at any dilution.

2.6. Investigation of immune protection in zebrafish model

Investigation of immune protection and cross-immune protection was carried out as described previously [42]. Zebrafish were randomly divided into a control and three experimental groups with twenty fish each group for immune protection and sixty fish each group for cross-immune protection. Each fish in the experimental groups was intraperitoneally injected with 5 μL of VPG suspension containing 1×10^6 or 1×10^7 VPG cells, and in control was injected with 5 μL of PBS. After 15 days, the zebrafish were challenged with 2 μL of *V. parahaemolyticus* at a concentration of 1.55×10^5 CFU and 2.42×10^4 CFU of *V. alginolyticus* through intraperitoneal injection to assess if they were protected by the vaccination of VPGs. The immune protective and cross-immune protective effects were assessed by relative percent survivals (RPS) of zebrafish at 15 days post immunization. Relative percent survivals calculated as RPS = 1- (%mortality of vaccinated group/% mortality of control group) × 100.

2.7. QRT-PCR for detection of innate immunity

QRT-PCR was carried out as described previously [42]. Zebrafish were randomly divided into experimental and control groups. The fish were anesthetized before intraperitoneal injection. Each fish was injected with 5 μL of VPG suspension containing 1×10^7 VPG cells as experimental groups, and with 5 μL of PBS as control.

These fish were cultured in 28 °C. After 24 h, the zebrafish spleens were obtained and grinded with cold liquid nitrogen before the total RNA was isolated with Trizol. The RNA was quantified by detecting the intensity of fluorescence. QRT-PCR was carried out on a PrimeScript™ RT reagent Kit with gDNA eraser (Takara, Japan) with 1 μg of total RNA according to manufacturer's instructions. QRT-PCR was performed in 384-well plates and each well contained a total volume of 10 μL liquid including 5 μL 2 SYBR Premix Ex Taq™, 2.6 μL PCR-grade water, 2 μL cDNA template and 0.2 μL each primer of forward and reverse (10 mM). The primers are listed in Table 1. All the samples were performed on LightCycle 480 system (Roche, Germany) according to the manufacturer's instructions and at least six independent samples were assayed for both control-group and experimental-group. To analyze the relative expression level of target genes, we converted the data to percentages relative to the value of control.

2.8. Cytotoxicity assay

The cytotoxic assays were performed as described previously [47]. HeLa cells (Thermo Fisher Scientific Inc.) were seeded in 96-well plates. The overnight cultured strains were sub-cultured at the ratio 1:50 to the fresh LB-NaCl broth medium and grow at 37 °C to $OD_{600} \approx 0.5$. Bacteria were pelleted by centrifugation and washed with PBS, then suspended in two volumes of serum-free DMEM. Lyophilized VPG or rVPG cells were suspended in two volumes of serum-free DMEM. Before infection, HeLa cells were also washed with serum-free DMEM. Infection was performed at an MOI of 10. After infection, the release of lactate dehydrogenase (LDH) into the medium was quantified at each indicated time point with a CytoTox 96 kit (Promega, Madison, WI, United States) according to the manufacturer's instructions.

3. Results

3.1. Production and characterization of VPGs

To generate *V. parahaemolyticus* ghosts (VPGs), we constructed P_{BAD}-gene E on a shuttle plasmid in which the expression of gene E can be induced under control of araBAD promoter (Fig. 1A). By transferring this plasmid into *V. parahaemolyticus* RIMD2210633 strain, we obtained a *V. parahaemolyticus* strain that can express Phi X174 protein E upon arabinose induction. Induction of the production of gene E by the addition of arabinose to the culture medium in mid-log phase resulted in a rapid protein E-mediated lysis in *V. parahaemolyticus* harboring P_{BAD}-gene E plasmid. To confirm the lysis efficiency, bacterial cells in series dilution concentration were spread on agar plate, and we found that gene E lysed 99.99% of *V. parahaemolyticus*. In order to achieve complete killing, the collected VPGs were subjected to lyophilization, and no living bacteria was found on plates at any dilution.

By scanning electron microscopy, *V. parahaemolyticus* ghost cells maintained intact cells but displayed modification of the cell envelope, likely the hole formed by protein E (Fig. 1B). Transmission electron micrographs also clearly showed that the cellular morphology of VPGs was not affected by lysis events, except for the lack cytoplasmic contents in the ghost cells (Fig. 1C).

3.2. Immune protective ability of VPG vaccine in the zebrafish model

Using the zebrafish model, the immune protection of VPG was investigated. We first tested the toxicity of VPGs to zebrafish. We found that VPGs were non-toxic as *D. rerio* injected with 1×10^6 or 1×10^7 VPG cells stayed healthy for over 15 days. The immunized *D. rerio* were then challenged with *V. alginolyticus* or *V. parahaemolyticus*. VPG showed stronger protective ability against the infection by either of these pathogens. Zebrafish immunized by VPGs showed over 80% survival (RPS) against infection by *V. alginolyticus* or *V. parahaemolyticus* respectively (Table 2). Yet, there is almost no difference in terms of RPS against *V. parahaemolyticus* for zebrafish immunized by VPG once or twice, but the immune protective ability against *V. alginolyticus* increased for those *D. rerio* immunized by VPGs twice compared to those one-time immunization (Table 2). These results indicated that VPGs was able to induce immune protection for *D. rerio* against infection by either *V. alginolyticus* or *V. parahaemolyticus*. Thus, VPGs could be the candidates of polyvalent vaccines.

3.3. Overexpression of outer membrane proteins, VP1667 and VP2369, in V. parahaemolyticus

We previously found that outer membrane proteins, VP1667 and VP2369, of *V. parahaemolyticus* could efficiently stimulate innate immune responses in zebrafish and provided zebrafish cross-immune protections against four pathogens [48]. To find out if the immune protection would be enhanced by overexpressing VP1667 and VP2369 in VPGs, we constructed a shuttle plasmid in which VP1667 and VP2369 fused with FLAG-tag and 6 x His-tag at their C-terminal respectively were under the control of LacI. The plasmid was then induced into the bacteria expressing the lysis gene E and resultant bacteria produced protein E and VP1667 and VP2369 upon the addition of arabinose and IPTG (Fig. 2A). Western blotting showed that the protein levels of VP1667 and VP2369 in recombinant VPGs (rVPGs) were much higher than that in wild type strain (Fig. 2B), which indicated that both VP1667 and VP2369 could be expressed at a high level in rVPGs.

3.4. Protective immunity of rVPGs vaccine in the zebrafish model

To test the immune protection of rVPGs in zebrafish against infection by *V. alginolyticus* or *V. parahaemolyticus*, rVPGs were administrated to zebrafish by i. p. injection and the vaccinated zebrafish were challenged by *V. alginolyticus* or *V. parahaemolyticus* at two weeks post vaccination. Like VPGs, rVPGs did not show any toxicity to zebrafish. rVPGs-vaccinated *D. rerio* showed over 80% of RPS, which is even higher than that of one-time immunization by VPG. In contrast, challenge of the PBS-injected control group resulted in at least 50% mortality (Table 3). Taken together, our results indicated that rVPGs has a better immune protective ability.

3.5. Innate immune response of zebrafish to rVPGs

To explore the potential role of innate immunity leading to the cross-protection in *D. rerio* upon the vaccination by rVPGs, we analyzed the transcriptional levels of innate immune genes of *D. rerio* by QRT-PCR. The results showed that rVPGs effectively stimulated the innate immunity in *D. rerio*. The expression of cytokines including *c3b, il1b, il6, il8, il10* and *tnfa* genes were significantly increased within 24–48 h after immunization with rVPG (Fig. 3A). The increased expression of *tlr5* and *lyz* genes were also detected in rVPGs immunized *D. rerio* (Fig. 3B). These results indicate that rVPG can effectively stimulate the immune responses in zebrafish.

3.6. Cytotoxicity analysis of VPGs and rVPGs

Our results showed that both VPGs and rVPGs produced efficient protection against at least two Vibrio pathogens. To test if VPGs and rVPGs that we generated in this study would be toxic to mammal cells, we compared the cytotoxicity induced by VPGs or rVPGs and *V. parahaemolyticus* wild type strain in HeLa cells. The lysis of HeLa cells was measured by monitoring the release of lactate dehydrogenase (LDH) after infection with either VPGs, rVPGs or *V. parahaemolyticus* wild type cells respectively. The result showed that HeLa cells were nearly completely lysed by *V. parahaemolyticus* wild type strain after 3 h infection. However, the cytotoxicity produced by either VPGs or rVPGs was much less than that produced by wild type strain when the same number of cells as that of wild type were used (Fig. 4). This suggested that neither VPGs nor rVPGs have any toxicity to the mammalian cells.

4. Discussion

Non-living micro-organisms have been widely used to induce protective immunity against microbial pathogens in vaccinology. Inactivated bacterial vaccines can efficiently stimulate immune response, yet their safety is a big concern when administered parenterally [22]. On the other hand, vaccines generated by purified components of many micro-organisms are often less immunogenic and require efficient adjuvants to be effective. Bacterial ghost produced by controlled expression of cloned lysis gene E of bacteriophage PhiX174 have been proved to be an efficient delivery vector that targets antigen component to the immune system and provides intrinsic adjuvant activity [27,29]. Bacterial ghosts of several pathogens, including *V. cholerae*, *Helicobacter pylori*, *Salmonella typhi* Ty21a and *E. coli*, were generated and used as vaccines or carriers for foreign target antigens, [36,49–51]. In this study, we successfully produced *V. parahaemolyticus* ghosts with high lysis efficiency by cloning lysis gene E into an *E. coli-V. parahaemolyticus* shuttle vector under the control of araBAD promoter. The lysis efficiency of VPGs or rVPGs reached more than 99.99% at 9 h after induction of gene E expression and there is no detectable living bacterial cells in lyophilized VPGs or rVPGs. Both scanning and transmission electron microscopic studies showed that VPGs were structurally intact and most cytoplasmic contents have been leaking out of the cells (Fig. 1). Park et al. generated VPGs by chemically-induced lysis and the VPGs prepared in this way showed similar cytotoxic activity as that of wild type bacteria [52]. However, VPGs and rVPGs generated by the inducible lysis E gene in this study showed much less cytotoxicity to HeLa cells and they were proved to be safe for zebrafish. This result might indicate that VPGs or rVPGs could work as effective antigens that induced the immunity protection in zebrafish.

Both VPGs and rVPGs showed efficient immunity protection in zebrafish against infection of at least two species of *Vibrio* species challenge, namely, *V. alginolyticus* and *V. parahaemolyticus*. Two outer membrane proteins, VP1667 and VP2369 that were overexpressed in rVPGs, are proved to be the polyvalent subunit vaccines in zebrafish model against infections by four microbe pathogens, including *V. alginolyticus, V. parahaemolyticus*, *Pseudomonas fluorescens* and *Aeromonas hydrophila*. The rVPGs generated in this study provided immunity protection for zebrafish even better than VPGs did. This is probably because that VP1667 and VP2369 overexpressed in rVPGs could increase the immunogenicity of rVPGs in zebrafish. Like VP1667 and VP2369, rVPGs can also efficiently stimulate innate immune response in zebrafish (Fig. 3). All these indicate that rVPGs is a promising candidate for polyvalent vaccine against infection of marine pathogens.

CRediT authorship contribution statement

Shengle Ji: Conceptualization, Methodology, Investigation. **Qiyang Gong:** Methodology, Investigation. **Wenwen Zhang:** Visualization, Investigation. **Jun Zheng:** Data curation, Writing - original draft, preparation. **Bo Peng:** Writing - Review & Editing, Supervision. **Menghua Yang:** Supervision, Project administration.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported by the Natural Science Foundation of China (31770151); the Science Development Foundation of Zhejiang A & F University (2013FR012) and the Natural Science Foundation of Zhejiang Province (LZ20C010001). The funding body had no role in the design of the experiments or the interpretation of the data.

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