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Generation and immunity effect evaluation of biotechnology-derived *Aeromonas veronii* ghost by PhiX174 gene *E*-mediated inactivation in koi (*Cyprinus carprio koi*)

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ABSTRACT

Aeromonas veronii is a conditional pathogen causing high mortality in many freshwater fish species worldwide. Bacterial ghosts are nonliving Gram-negative bacteria devoid of cytoplasmic contents, which induce protective immunity against microbial pathogens. The aims of this study were: a) to produce *A. veronii* ghost (AVG) constructed by PhiX174 gene *E*; b) to evaluate the specific, non-specific immune effects and protective immunity of AVG against *A. veronii* in koi. The lysis plasmid pBBR-E was constructed by cloning PhiX174 gene *E* into the broad-host-range vector pBBR1MCS2, and then transformed into *A. veronii* 7231. AVG was generated by increasing the incubation temperature up to 42 °C. Lysis of *A. veronii* occurred 3 h after temperature induction and completed in 12 h. The efficiency of ghost induction was 99.9998 ± 0.0002%. Koi were immunized intraperitoneally with AVG, formalin-killed bacteria (FKC) or phosphate buffered saline (PBS) respectively, and then respiratory burst (RB), myeloperoxidase (MPO), lysozyme (LZM), malondialdehyde (MDA), complement 3 (C3) and antibody activities were examined in serum. Compared with negative control of PBS, the RB, MPO, LZM activities were significantly higher in koi immunized with AVG ($P < 0.05$). Nevertheless, the MDA activities of AVG treatment were significantly lower than those of PBS treatment ($P < 0.05$). The serum agglutination titers and IgM antibody titers in AVG group were significantly higher than those in FKC or PBS groups. After challenged with the parent strain *A. veronii* 7231, the average mortality of AVG group was significantly lower than that of FKC and PBS groups ($P < 0.05$) and the relative percent survival (RPS) of AVG group (73.92%) was higher than that of FKC group (43.48%). Therefore, AVG have the potential to induce protective immunity and they may be ideal vaccine candidates against *A. veronii* in koi.

1. Introduction

Aeromonas veronii, a Gram-negative bacterium, is a conditional pathogen widely distributed in nature, such as fresh water, sewage, sludge and soil. The diseases caused by *Aeromonas veronii* in carp, trout, ornamental fishes, catfish, loach and sturgeon lead to extensive losses worldwide [1–6]. Most of koi suffering from *A. veronii* disease have hemorrhage, skin rot and ulcer symptoms. Almost all skin ulcer koi are scarred and lose their ornamental value, which has brought great economic losses. So prevention and cure of this disease is crucial in aquaculture.

Antibiotics and disinfectors are used to treat fish diseases in recent years. However, the abuse of antibiotics and disinfectors has caused a lot of problems, such as emergence of antibiotic-resistant microbes [7,8], decline of fish immunity [9], drug residues *in vivo* [10–12] and so on. Over the last decade, vaccination has been important for prevention of infectious diseases in farmed fish [13]. The strategies to research bacterial vaccine in aquaculture include inactivated vaccine, attenuated vaccine, and gene-engineering vaccine. Live attenuated vaccine is limited because of safety identification to consumers and the environment [14]. Inactivated vaccine has been proved to be a safe vaccine. Formalin-killed bacteria (FKC) were one of most widely used vaccines made of inactive bacteria. It is safe but not efficient enough to elicit strong protection [15,16]. On the other hand, bacteria ghost has been

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given increasing attention as a novel inactivated vaccine by means of gene-engineering.

Bacterial ghost is empty and intact bacterial envelope of Gram-negative bacteria that is produced by controlled expression of the phage PhiX174 lysis gene *E* [17]. The protein E can form an E-specific transmembrane lysis tunnel, whose structure is restricted to areas of potential division sites, predominantly in the middle of the cell or at polar sites [18,19]. The remaining bacterial inner space is devoid of nucleic acids, ribosomes or other constituents, whereas the inner and outer membrane structures of the ghosts are well preserved [20]. In particular, they display all surface components in a natural form and are able to induce a strong immune response [21]. In this paper, we report generation method of biotechnology-derived *Aeromonas veronii* ghost, immunological responses and protection in koi (*Cyprinus carprio*) after being immunized with A. *veronii* ghost.

2. Materials and methods

2.1. Materials and reagents

2.1.1. Bacteria strains, media and plasmids

Aeromonas veronii 7231 was isolated from naturally ulcer koi (*Cyprinus carprio*) in a fish farm in Beijing, China, and it was stored in China General Microbiological Culture Collection Center (CGMCC). *Escherichia coli* used for the positive control were the commercially available DH5α strain (TransGen, Beijing, China). *Escherichia coli* S17-1 and plasmid pBRR1MCS2 were kindly provided by Dr. G. Q. Chen, Tsinghua University, China. *Escherichia coli* S17-1 was grown in Luria-Broth (LB, Difco, USA) liquid medium or on LB agar plates supplemented with spectinomycin (100 μg mL^{-1}) and used as a donar in conjugation of *A. veronii*. A shuttle vector pHH43 was used to amplify lysis gene cassette and was kindly provided by Dr. C.J. Liu, Beijing Institute of Biotechnology, China. Transformed *A. veronii* was selected on LB plates containing 50 μg mL^{-1} kanamycin and 100 μg mL^{-1} ampicillin. Growth and lysis of bacteria cultures were monitored by measuring the optical density at 600 nm (OD_{600}).

2.1.2. Reagents and kits in DNA manipulations

Plasmid pHH43 and pBBR1MCS2 were purified using a plasmid mini kit (Tiangen, Beijing, China). Plasmids and DNA fragments were digested under conditions described by the restriction enzymes manual (New England Biolabs, CA, USA). Restriction digests were visualized on 1% agarose gels stained with ethidium bromide. DNA fragments were purified using Gel and PCR Clean-up System (Promega, WI, USA). Recombinant plasmid construction was conducted on *E. coli* DH5α. *E. coli* cells for electroporation were prepared according to the protocol recommended for the Gene Pulser (BTX, Beijing, China).

2.2. Construction and preparation of A. veronii ghost (AVG) and formalin-killed A. veronii (FKC)

2.2.1. Construction of lysis vector

The lysis E cassette encoding lambda P_R-cI_{857} regulatory system and PhiX 174 lysis E gene was amplified by PCR using the plasmid pHH43 as the template and the primers lysisF (5′-CGGGATCCTCAGCCAAACG TCTCTTCAG-3′) and lysisR (5′-CGGGATCCTCACTCCTTCCGCACGT AAT-3′), which add *Bam*HI restriction site (underlined). PCR amplifications were performed as follows: initial denaturation at 95 °C for 3 min, followed by 30 cycles (95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min) and a final elongation at 72 °C for 10 min. The PCR product of lysis E cassette was inserted into the *pEASY*™-Blunt Zero Cloning Vector (T-vector, TransGen, Beijing, China). After sequencing, the lysis E cassette was inserted into the plasmid pBBR1MCS2 which was predigested with the same restriction enzyme. The resulting plasmid was designated as pBBR1MCS2-E.

2.2.2. Conjugation of A. veronii

The transformation of *E. coli* was performed as previously described [22]. The pBBR1MCS2-E plasmid was introduced by electroporation into the S17-1 strain of *E. coli*, and transformants were selected at 28 °C on LB plates containing 50 μg mL^{-1} kanamycin. *E. coli* S17-1 harboring pBBR1MCS2-E was used as a donor for conjugation with *A. veronii*. *E. coli* and *A. veronii* were inoculated into 40 mL LB and grew at 28 °C. At an OD_{600} of 0.5–0.7, donor and recipient cells were harvested by centrifugation at 8000*g* for 10 min and resuspended first in 25 mL, and then in 1 mL of LB medium to a density of 5×10^6 cells mL^{-1}. Equal volumes (400 μL) of donor and recipient cells were mixed and incubated at 28 °C for 24 h . After incubation the resulting cells were spread onto LB agar plates containing 50 μg mL^{-1} kanamycin and 100 μg mL^{-1} ampicillin.

2.2.3. Production of A. veronii ghosts (AVG)

A single colony of the *A. veronii* transformants from previous conjugation step, carrying pBBR1MCS2-E, was selected into 3 mL LB containing 50 μg mL^{-1} kanamycin. The cultures reached an OD_{600} of 0.4–0.6 at 28 °C. The expression of gene *E* was induced by a change in incubation temperature from 28 °C to 42 °C. OD_{600} values were examined every hour. At the end of lysis progress, 100 μL of the culture were inoculated on a LB plate in order to examine whether or not there were any survival cells. Efficiency of bacteria ghost induction was calculated as follows: lysis rate = (1 - CFU of lysis completed/CFU before induction) × 100%. The AVG were harvested by centrifugation (4 °C, 6000 g, 10 min), washed three times with PBS and resuspended in PBS to reach a bacterial concentration of 1×10^8 cells mL^{-1}.

2.2.4. Production of formalin-killed A. veronii (FKC)

A. veronii 7231 was inoculated for 16 h at 28 °C in LB. Formalin was added to the culture to make the final concentration reach 0.5%. After 48 h incubation, cells were washed three times with phosphate buffered saline (PBS, pH 7.2) and resuspended in PBS to a bacterial concentration of 1.1×10^8 cells mL^{-1}. The suspensions were spread on LB agar plates for checking inactivation effect, and the FKC was stored at 4 °C.

2.2.5. Electron microscopy analysis

Samples were fixed with 2.5% (wt/vol) glutaraldehyde and 2% (wt/vol) paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at 4 °C for 2 h. After rinsed in 0.1 M sodium cacodylate buffer for three times, samples were fixed in 1% (wt/vol) osmium tetroxide for 2 h at room temperature. For embedding, samples were dehydrated in a graded series of ethanol and embedded with Epon 812 resin according to the described protocol [23]. Ultrathin sections were counterstained with uranyl acetate and lead citrate before examination by transmission electron microscopy (TEM) in a Hitachi H-7650 at an acceleration voltage of 80 kV.

The samples were dropped and placed in room temperature for 1 h. The fixation of samples was performed by incubation in a 2.5% (wt/vol) glutaric dialdehyde solution for 30 min. The fixed samples were then dehydrated in a graded concentration of ethanol. The dehydrated cells were treated with tetra-butanol, put into a 60 °C incubator for 1 h and cooled down at −20 °C for 1 h. Freeze dried the samples for about 4 h–8 h until the samples were dried completely. Then samples were ready to be sputtered with gold-palladium and examined in a Hitachi S-4800 scanning electron microscope (SEM).

2.3. Immunization and challenge test

2.3.1. Immunization procedures

Koi (35 ± 5 g) were obtained from a commercial fish farm in Beijing, China. All fish were found to be free of parasitic and bacterial pathogens prior to the experiments. Fish were stocked into nine 260 L aquaria with aeration and acclimated for two weeks. Fish were fed commercial feeds twice on a daily basis at a 2% feeding rate. Water was

partly changed daily and the water temperature was kept at 23.5 ± 1 °C. Koi were randomly divided into three treatment groups (AVG, FKC and PBS), and each group had three duplications of 28 fish.

Each fish of AVG and FKC group was intraperitoneally (i.p.) injected respectively with 200 μL of AVG and FKC on day 0 and day 14. Each fish of PBS group was i. p injected with 200 μL PBS to serve as the control on day 0 and day 14.

2.3.2. Sample collection

Three fish in each tank were sampled randomly on day 7, 14, 21, 28, 35 and 42. Blood samples were taken by the caudal venipuncture after anesthetized with MS-222 (Sigma, USA). After collection, whole blood was allowed to clot at room temperature for 4 h. The serum was removed by centrifugation (3000 g, 10 min, 4 °C), and frozen at −80 °C until use. The blood samples of day 7, 14, 21 and 28 were used to test nonspecific immunity parameters. All blood samples were used to test specific immunity parameters.

2.3.3. Challenge test

On day 42, all three groups were challenged via intramuscular injection (i.m.) with 3×10^6 cells of *A. veronii* 7231 in 200 μL of PBS. The fish were monitored for 14 days after challenge, and the cumulative percent mortality (CPM) was calculated for each group. Relative percent survival (RPS) was calculated as previously described [24], RPS = (1 - immunized group mortality/control group mortality) × 100%.

2.4. Analysis of serum enzyme activity

2.4.1. Respiratory burst activity (RB)

The respiratory burst activity of phagocytes was carried out using the nitroblue tetrazolium (NBT, Shanghai Reagent Corp., China) assay following the method of Anderson and Siwicki [25] and subsequently modified by Sahoo [26]. Briefly, 100 μL of whole blood anticoagulated by heparin was added to 100 μL of 0.2% NBT (Sigma, USA) solution in tube. The mixture was incubated for 30 min at room temperature. 45 μL of suspension and 900 μL of N,N-dimethyl formamide (DMF; Beijing Borunlaite science & technology Co., Ltd, China) were mixed utterly, and then centrifuged at 3000 g for 5 min. The OD of the supernatant was read by an automatic plate reader at 540 nm.

2.4.2. Myeloperoxidase activity (MPO)

Total MPO activity in serum was measured according to Quade and Roth [27] with slight modification. About 10 μL of serum was diluted with 90 μL of Hanks balanced salt solution (HBSS) without Ca^{2+} or Mg^{2+} in 96-well plates. Then 35 μL of 20 mM 3,3′,5,5′-tetramethylbenzidine hydrochloride (TMB) (Sigma, USA) and 5 mM H_2O_2 (both substrates of MPO and prepared on same day) were added. The color change reaction was stopped after 2 min by adding 35 μL of 4 M sulphuric acid (H_2SO_4). The OD was read by an automatic plate reader at 450 nm.

2.4.3. Malondialdehyde content (MDA)

The MDA content was measured by its ability to condense with thiobarbituric acid (TBA), and generating red degradation products [28] by using an MDA detection kit (Nanjing Jiancheng Bioengineering Institute, China). The OD was measured at 532 nm in a microplate reader.

2.4.4. Lysozyme activity (LZM)

Lysozyme activity in serum was measured by turbidimetric assay according to the method of Ellis [29]. Briefly, 100 μL of serum was added to 1.9 mL of a suspension of *Micrococcus lysodeikticus* (Sigma) (0.2 mg mL^{-1}) in a 0.05 M sodium phosphate buffer (pH 6.2). The reaction was carried out at 25 °C and absorbance was measured at 530 nm after 0.5 and 4.5 min in a spectrophotometer. One unit of lysozyme activity was defined as the amount of sample causing a reduction in absorbance of 0.001 min^{-1}.

2.4.5. Complement 3 content (C_3)

The serum complement 3 (C_3) level was determined using commercial test kits (Weiyi Bioengineering Institute, Zhejiang, China). Briefly, the C3 of serum sample was bound with its antibody in the kit and generated to an antigen-antibody complex, increasing in turbidity. The OD was measured at 340 nm in a microplate reader.

2.5. Analysis of antibody titers

2.5.1. Agglutination activity of serum

The agglutination test was conducted in 'U'-shaped microtiter plates. The test sera were used the doubling dilution to the eighth tube with sterile PBS. 50 μL of serial dilution sera and the same volume of *A. veronii* 7231 (3×10^7 cells mL^{-1}) was added to each well of 96-well plates. Negative sera and bacteria were added respectively in every plate as controls. The plates were incubated overnight at 28 °C in moist surrounding. A circular diffuse deposition with blurry edges at 'U'-shaped bottom was judged as positive reaction; and clear compact circular deposition was determined as negative reaction. The agglutination titers were defined as the reciprocal of the highest dilution.

2.5.2. Analysis of IgM titers in serum by ELISA

Titers of *A. veronii* antigen-specific immunoglobulin M (IgM) were determined by indirect ELISA described previously [30]. Plates were incubated with HRP-conjugated rabbit anti-carp IgM isotype (prepared in this lab) for 1 h at 37 °C and developed with (3,3′,5,5′-tetramethylbenzidine) (TMB). The OD was measured at 450 nm on a microplate reader. Results were calculated by the ratio of OD from sample and negative control, and the standard of the positive is or above 2.1. The largest dilution judged as positive was recorded as the IgM titers of serum by ELISA.

2.6. Statistical analysis

Statistical analysis was carried out using SPSS 11.0 for Windows. All data were presented as mean ± S.E.M. and analyzed by one-way analysis of variance (ANOVA) and Duncan's test to determine significant differences. An overall significance level of $P < 0.05$ was accepted.

3. Results

3.1. Construction of shuttle lysis vector

The lysis *E* cassette was obtained by PCR from the plasmid pHH43 which is a shuttle plasmid of *Helicobacter pylori-E. coli*. The gene *E* cassette, which was 1300 bp in length, was ligated into the broad-host-range (bhr) cloning vector pBBR1MCS2. Therefore the *E. coli-A. veronii* shuttle lysis vector, pBBR1MCS2-E, was constructed (Fig. 1.). This vector harbored kanamycin resistance, since *A. veronii* 7231 was highly sensitive to kanamycin but resistant to ampicillin.

3.2. Generation and characterization of A. veronii 7231 ghost

A. veroni 7231 was transformed by conjugation with donor, *E. coli* S17-1 harboring pBBR1MCS2-E. Transformants were propagated and lysis was induced at mid-log phase by increasing the incubation temperature up to 42 °C (Fig. 2.). Outset of lysis was detected from 3 h post-induction of gene *E* by monitoring the OD_{600}. End of lysis was observed 12 h after gene *E* induction. The concentration of viable organism dropped from 1 to 2.6×10^8 cells mL^{-1} to 0–2.4×10^3 cells mL^{-1}. At the end of the lysis process, the efficiency of *A. veronii* ghost was 99.9998 ± 0.0002% in 5 replicate experiments.

It was observed that the *A. veronii* ghosts had the structural integrity

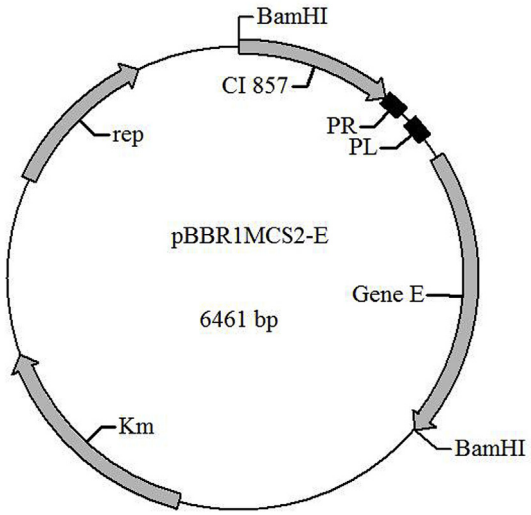

Fig. 1. A shuttle lysis vector pBBR1MCS2-E. Gene *E*, under transcriptional control of the temperature-sensitive repressor; PR/PL: the regulating sequence including promoter; rep: replication origin; CI 857: restraining gene of lambda bacteriophage adapted to heat induced expression.

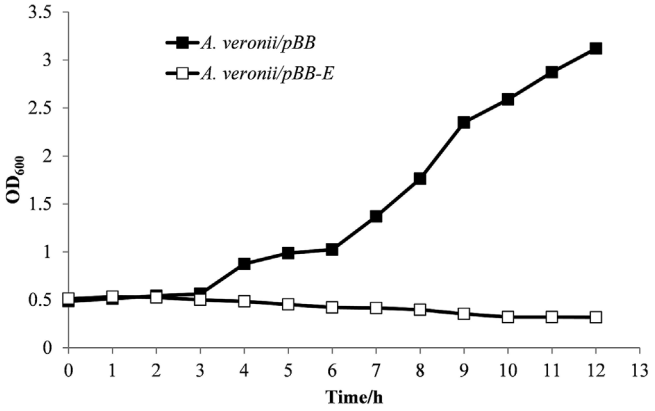

Fig. 2. Growth curves of *A. veronii* 7231 and lysis curves of its harboring plasmid pBBR1MCS2-E by temperature induction of gene *E* expression. At time zero, the incubation temperature of cultures was shifted from 28 °C to 42 °C.

of bacterial outer membrane except for the lysis pore, but the cells was obviously shrinking because the intracellular substances had been released through the pores on the ends of bacteria (Fig. 3(a) and (b)). Pores of 100 nm in diameter were observed in AVG by SEM.

3.3. Nonspecific immunity

3.3.1. Respiratory burst activity

The respiratory burst activities of AVG and FKC treat group were significantly higher than that of PBS group ($P < 0.05$) on day 21 (Table 1). No significant differences in respiratory burst activity were observed in three experimental groups on day 7, 14, 28.

3.3.2. MPO activity

The MPO activity of AVG treatment was significantly higher ($P < 0.05$) than that of FKC and PBS treatment on day 28 (Table 2). No significant differences in MPO activity were observed in three experimental groups on day 7, 14 and 21.

3.3.3. MDA activity

Compared with PBS treatment, the MDA activity of serum was significantly higher ($P < 0.05$) in koi injected with FKC on day 14 (Table 3). On day 28 the serum MDA of AVG and FKC was significantly lower ($P < 0.05$) than that of PBS.

3.3.4. Lysozyme activity

Compared with the negative control group of PBS, the lysozyme activity of serum was significantly higher ($P < 0.05$) in koi immunized with AVG and FKC on day 28 (Table 4).

3.3.5. Complement 3 activity

There was no significant difference in C_3 activity among those three groups ($P > 0.05$) (Table 5).

3.4. Specific immunity

3.4.1. Agglutination titers

It was showed that the serum agglutination titers in AVG and FKC groups were significantly higher ($P < 0.05$) than those in PBS group on day 7, 14, 21, 28, 35 and 42 (Fig. 4). Furthermore, the titers of AVG group were significantly higher ($P < 0.05$) than those of FKC group on day 7, 35 and 42. The agglutination titers of serum in AVG group significantly increased on day 35 and 42, and the peak of agglutination titer was $2^{6.57}$. The agglutination titers of the serum in FKC group reached the highest value (2^4) on day 42 (Fig. 4.).

3.4.2. IgM titers tested by ELISA

The IgM titers of AVG and FKC group tested by ELISA were significantly higher ($P < 0.05$) than those of the PBS group on day 14, 28, 35 and 42 (Fig. 5), and IgM titers of AVG group were significantly higher ($P < 0.05$) than those of FKC group on day 35. In AVG group, the IgM titers tested from day 28–42 were significantly higher ($P < 0.05$) than those tested at time points before day 28, and the highest titer was 2^{11} on day 35. In FKC group, the IgM titers tested on day 28, 35 and 42 were significantly higher ($P < 0.05$) than those tested on day 7 and 14, and the peak of the IgM titer was $2^{9.87}$ on day 28.

3.5. Protective efficiency of AVG vaccine

The RPS of test groups was shown in Table 6. Among the three groups, AVG has the highest survival rate after challenge. The average cumulative mortality of AVG was 25 ± 7.22%, with RPS being 73.92%. In contrast, the RPS of FKC treat group was only 43.48%, which is lower than that of AVG. In addition, most dying fish showed typical clinical signs of ulcer disease. Colonies of *A. veronii* were recovered from all dead fish.

4. Discussion

In this work, *A. veronii* ghosts (AVG) were generated by the temperature-controlled expression of lysis gene *E*. The lysis pore was observed by transmission electron microscopy (TEM) and scanning electron microscopy (SEM). Bacterial ghosts have been produced from a variety of bacteria in fisheries, including *Edwardsiella tarda* [31], *Aeromonas hydrophila* [32], *Flavobacterium columnare* [15], *Vibrio anguillarum* [33] and *Vibrio alginolyticus* [34]. The efficiencies of bacterial ghosts induction in *E. tarda*, *A. hydrophila*, *F. columnare*, *V. anguillarum* and *V. alginolyticus* were 99.99 ± 0.01%, 99.99%, 99.99%, 100% and 99.91%, respectively. The efficiency of AVG generation (99.9998 ± 0.0002%) was better than most of the ghosts mentioned above. Observed by electron microscope, the morphology structure of AVG was also the same as those of the other bacterial ghosts in fish.

Bacterial ghosts, being a vaccine candidate, has been studied in many mammals [35,36] and also in several aquatic animals [15,31,32]. However, most workers focused on the specific immunity of fish vaccinated with bacterial ghosts [37–40]. There have been few reports on the non-specific immunity in fish. Especially *A. veronii* ghost has not

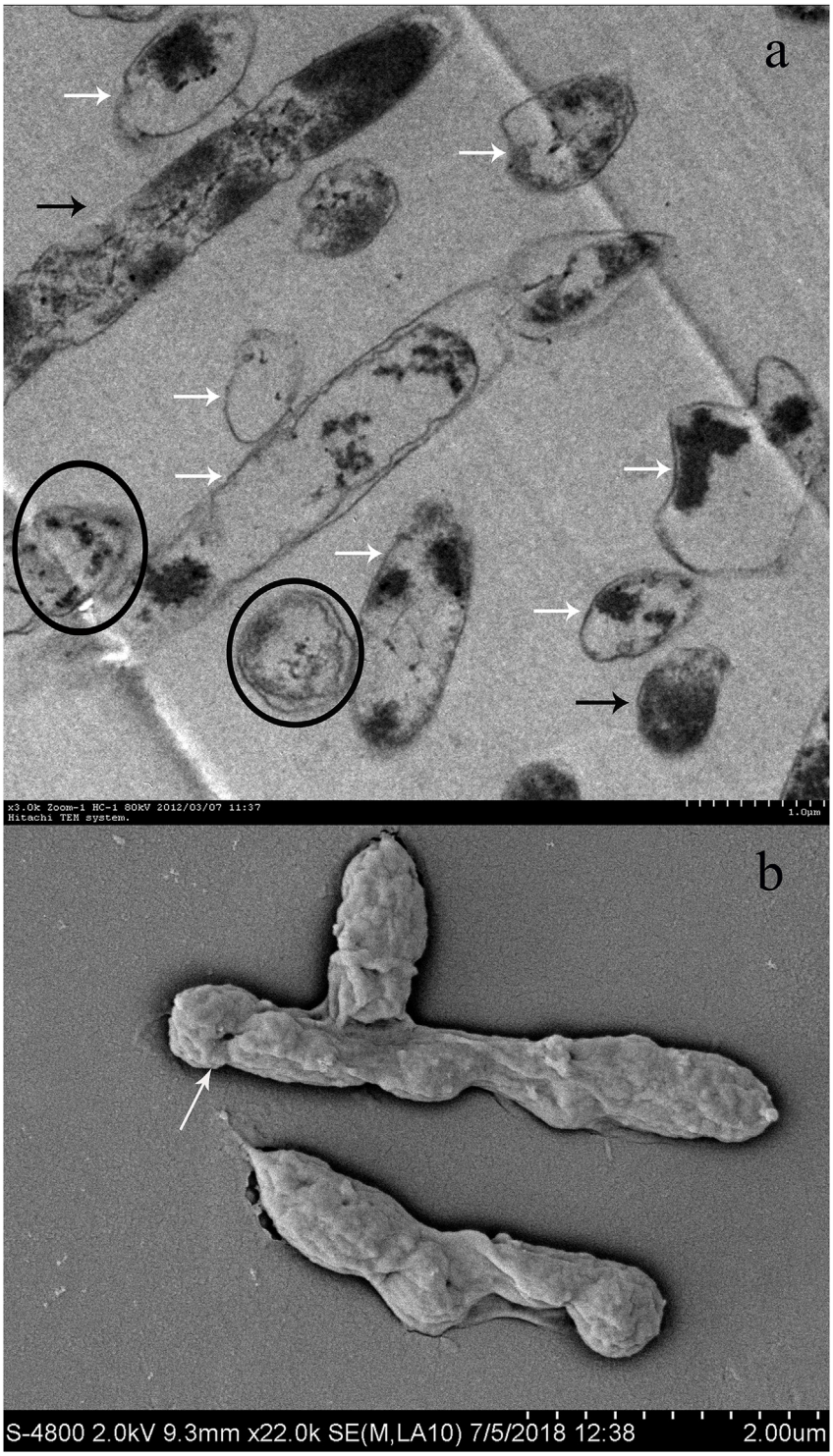

Fig. 3. TEM (a) and SEM (b) analysis of *A. veronii* lysed by the induction of the lysis *E* gene. (a) Loss of intracellular substances of *A. veronii* 7231 ghosts. The lysed cells (white arrow) showed uneven and low electron density, while the unlysed cells (black arrow) showed even and high electron density. The wall of bacteria ghosts showed shrinkage (circle) because of the loss of cytoplasmic materials. (b) Transmembrane lysis tunnel (white arrow).

been reported up to date. Nevertheless, there are two reasons that it is necessary to study non-specific immunity of bacterial ghost in fish. Firstly, the non-specific defense mechanism in lower vertebrates, for example fish, plays an important role at all infection stages. The non-specific humoral defense including protease, lysins and agglutinins is the first line of defense [41]. Secondly, the bacterial ghosts retain the functional and antigenic determinants of the envelope with their living counterparts. Therefore, they possess good immunogenicity and adjuvant properties [34].

As well known, the non-specific immunity of fish is gained from two ways on the level of cellular immunity of phagocytes. The first one is an oxygen dependent kill mechanism, which depended on the reactive oxygen intermediates (ROI) or reactive nitrogen intermediates (RNI) [42,43]. In this study, levels of respiration burst, MPO and MDA are related to the first aspect. The second way is an oxygen independent kill mechanism, which refers to the various kinds of enzymes that can not

Table 1
The respiratory burst activity of koi immunized with experimental vaccines.

	7 d	14 d	21 d	28 d
AVG	0.215 ± 0.013	0.218 ± 0.007	0.229 ± 0.006^{b}	0.217 ± 0.004
FKC	0.211 ± 0.013	0.215 ± 0.013	0.231 ± 0.009^{b}	0.223 ± 0.012
PBS	0.196 ± 0.006	0.222 ± 0.008	0.198 ± 0.008^{a}	0.210 ± 0.005

Means in the same rank with different superscripts are significantly different ($P < 0.05$).

Table 2
The MPO activity of koi immunized with experimental vaccines.

	7 d	14 d	21 d	28 d
AVG	51.86 ± 5.48	61.06 ± 5.34	54.17 ± 7.22	38.00 ± 6.73^{b}
FKC	42.51 ± 2.56	52.46 ± 5.42	67.81 ± 6.12	22.46 ± 1.35^{a}
PBS	56.77 ± 5.67	55.18 ± 2.53	65.83 ± 7.09	25.49 ± 1.69^{a}

Means in the same rank with different superscripts are significantly different ($P < 0.05$).

Table 3
The MDA activity of koi immunized with experimental vaccines.

	7 d	14 d	21 d	28 d
AVG	6.69 ± 0.40	6.46 ± 0.29^{ab}	6.05 ± 0.16	6.31 ± 0.39^{a}
FKC	6.47 ± 0.37	6.86 ± 0.35^{b}	6.23 ± 0.33	6.16 ± 0.52^{a}
PBS	7.11 ± 0.32	5.87 ± 0.21^{a}	6.45 ± 0.40	7.71 ± 0.34^{b}

Means in the same rank with different superscripts are significantly different ($P < 0.05$).

only hydrolyze whole pathogens but also digest and degrade furtherly. Lysozyme tested in this research is concluded in the second aspect [41]. In addition to these immune parameters mentioned above, there is still an important serial of non-specific immune factor, which is the complement system and it has also been observed in this study.

Respiration burst takes place when phagocytes such as monocytes, macrophages, and neutrophil granulocytes swallowing pathogens in fish. Thereafter, NADPH-dependent oxidase located in plasma membrane is activated to generate superoxide anion radical [44], and such reactive oxygen species have a strong bactericidal effect independently or associated with other bactericidal systems [45], such as MPO-H_2O_2-halide system [46]. Compared with the negative control group, koi immunized with AVG or FKC revealed a higher NBT activity. Similar result was also achieved by Dash S et al., who found Indian major carp (*L. rohita Ham*) injected intraperitoneally with formalin killed *Aeromonas hydrophila* has shown a remarkably enhanced NBT parameter [26]. This result suggested that AVG elicited cellular immunity of phagocytes to eliminate the bacteria.

MPO is an important enzyme having antimicrobial activity. It utilizes hydrogen peroxide during respiratory burst to produce hypochlorous acid [43]. Reduced activity may indicate the presence of contaminants or stress [25]. To the best of our knowledge, this was the first time to test MPO activity of koi vaccine with bacterial ghost. In this study, a comparatively higher level of MPO activity was found in koi immunized with AVG compared with the FKC and PBS groups. Since MPO was directly related to neutrophil number, it was widely used to

Table 5
The C_3 activity of koi immunized with experimental vaccines.

	7 d	14 d	21 d	28 d
AVG	0.057 ± 0.003	0.059 ± 0.002	0.056 ± 0.002	0.059 ± 0.002
FKC	0.059 ± 0.001	0.053 ± 0.002	0.052 ± 0.003	0.055 ± 0.003
PBS	0.057 ± 0.002	0.058 ± 0.002	0.056 ± 0.003	0.057 ± 0.001

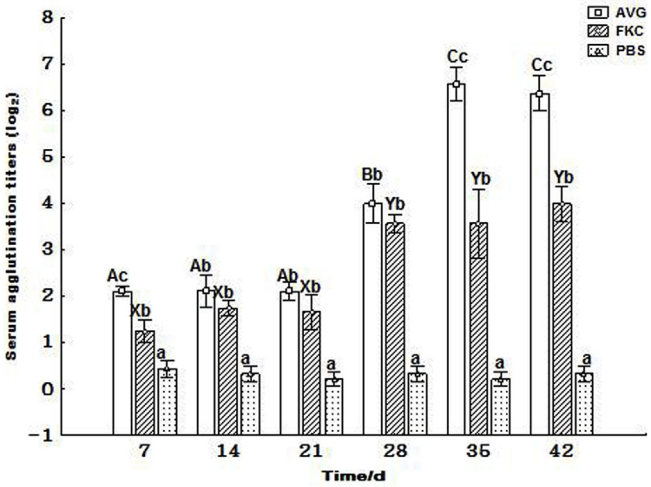

Fig. 4. Antibody titers tested by agglutination in koi vaccinated with AVG, FKC or PBS. Values are mean ± SE. Different capital letters on the bar indicate statistically significant differences among different time points in the same treatment group. Different lowercase letters on the bar indicate statistically significant differences among different treatment groups at the same time point.

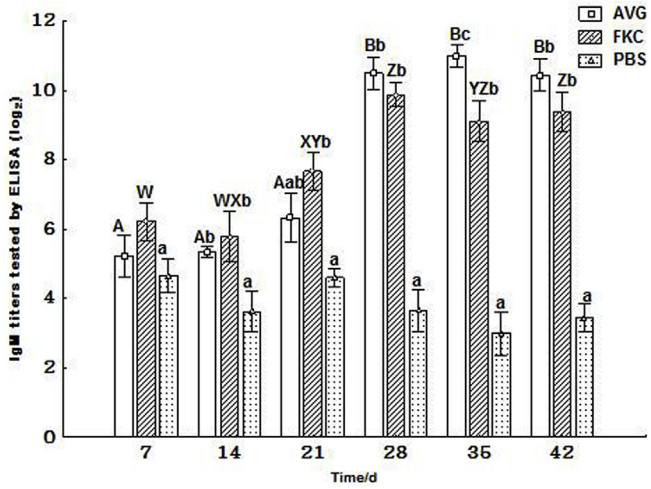

Fig. 5. IgM titers tested by ELISA in koi vaccinated with AVG, FKC or PBS. Values are mean ± SE. Different capital letters on the bar indicate statistically significant differences among different time points in the same treatment group. Different lowercase letters on the bar indicate statistically significant differences among different treatment groups at the same time point.

Table 4
The lysozyme activity of koi immunized with experimental vaccines.

	7 d	14 d	21 d	28 d
AVG	333.19 ± 32.02	272.44 ± 11.72	560.69 ± 33.34	625.63 ± 17.75^{b}
FKC	335.51 ± 36.67	304.12 ± 22.45	607.30 ± 34.20	566.34 ± 32.48^{b}
PBS	273.57 ± 15.12	351.64 ± 57.55	553.38 ± 23.74	258.21 ± 29.54^{a}

Means in the same rank with different superscripts are significantly different ($P < 0.05$).

Table 6
The mortality and relative percent survival (RPS) of challenged koi (*Cyprinus carprio*) vaccinated with *Aeromonas veronii* ghosts (AVG), formalin-killed *A. veronii* (FKC) or PBS (Control).

groups	challenge number	mortality	mortality rate (%)	average mortality rate (%)	RPS (%)
AVG	8	1	12.50	25.00 ± 7.22[a]	73.92
	8	2	25.00		
	8	3	37.50		
FKC	8	5	62.50	54.17 ± 4.17[b]	43.48
	8	4	50.00		
	8	4	50.00		
PBS	8	8	80.00	95.83 ± 4.17[c]	0
	8	8	80.00		
	8	10	100.00		

Notes: Different superscripts indicate significantly different values ($P < 0.05$).

be a marker for tissue neutrophil content [47]. Wang reported that the number of neutrophils significantly increased after immunization with bacterial ghosts, *Edwardsiella ictaluri* ghosts [17]. Therefore, the significant increase of MPO in AVG group might result from the increase of neutrophil number.

MDA is a product of lipid peroxidation, which can be used to evaluate the oxidative damage in lipids [48,49]. In fish, an excess of reactive oxygen species (ROS) causes oxidative damage to lipids, which lead to DNA, cell, and tissue damage [50]. To prevent this damage, there should be a balance between ROS generation and antioxidant defense [51]. Since MDA is a substance with strong biotoxicity [52], lipid peroxidation and oxidative stress in fish are diagnosed by monitoring MDA level [53], [53]. In the present study, serum MDA of AVG treatment was significantly lower in comparison with that of negative control of PBS. This finding was in accordance with effects of dietary mannan oligosaccharide on crucian carp, *Aloe vera* on tilapia and protein levels on grass carp [54–56]. It was reported that polysaccharides from marine bacterium *Edwardsiella tarda* exhibited antioxidant activity, especially inhibition of lipid peroxidation [57]. Therefore, the significant difference in serum MDA between AVG and PBS probably derived from polysaccharide that existed in inactivated *Aeromonas veronii*. These results revealed that AVG protected koi from oxidative damage.

Lysozyme (N-acetylmuramide glycabhydrikase), which is secreted by leucocytes that are distributed in various tissues and the circulation [58–60], cleaves the glycosidic bonds in the peptidoglycan layer of certain Gram-positive bacteria. Lysozyme also has antimicrobial activity against several Gram-negative bacteria [61,62]. The activity of lysozyme in koi vaccinated with AVG and FKC is significantly higher compared with the negative control. Similar results were observed in carp injected with formalin-killed *Aeromonas bestiarum* [63] and rainbow trout vaccinated with live attenuated *Aeromonas hydrophila* [64]. The elevation of lysozyme was likely due to the increased generation of neutrophils in the peripheral blood, since peritoneal macrophages and blood neutrophils contained lysozyme and the latter was thought to be the source of serum lysozyme [65]. In Wang's paper, the number of neutrophils significantly increased after immunization with *Edwardsiella ictaluri* ghosts [17]. This result suggested that bactericidal effect in fish was activated by AVG.

The complement system is composed of a number of proteins found in the serum. Complement acts as a link between the humoral and cell-mediated specific immune responses. Moreover, complement also plays an important role in non-specific host defense mechanism [66]. In particular, complement 3 (C3) is a key molecule in the complement system and its activation is essential for all the important functions performed by this system. In this study, the changes of C3 content in koi showed no significant difference among the 3 treatment groups on day 7, 14, 21, 28. Therefore, it was inferred that C3 was not activated by AVG at those time points.

Some reports have indicated that bacterial ghosts can elicit specific immune responses [17]. In this study, koi immunized intraperitoneally with AVG revealed significantly higher serum agglutination antibody titers and IgM titers than those immunized with both formalin-killed *A. veronii* (FKC) and PBS. Similar results were observed in live-attenuated *A. hydrophila* immunized rainbow trout [67] and live-attenuated *A. hydrophila* [68] injected common carp. Moreover, the specific antibody titers of koi immunized with AVG group were higher than those of other bacterial ghosts groups [17,38,39]. On one hand, in the present study koi were injected intraperitoneally at the dose of 2×10^7 cells, while olive flounder and *Carassius auratus gibelio* were fed orally with 4×10^8 cells and 5×10^8 cells, respectively [38,39]. It was probably because fish vaccines were more effective when delivered by injection rather than orally. On the other hand, koi were vaccinated twice during 14 d, while catfish were vaccinated only once in Wang's study [17] with the same dose of the A. *veronii* ghosts. Therefore, the number of vaccination was another factor that impacted on specific immune effect. In addition, Dash [69] reported that a single dose of 10^{10} cells mL^{-1} of formalin killed *Aeromonas hydrophila* could elicit the highest antibody response among three different doses (10^5 cells mL^{-1}, 10^7 cells mL^{-1}, 10^{10} cells mL^{-1}). The different doses of vaccination probably resulted in the different specific immune effect between AVG and other bacterial ghosts groups. Another important parameter of immune response, RPS value, was significantly higher in AVG group than FKC group and this result corresponded to those published previously [15,32,37–40]. It was reported that bacteria ghosts were empty envelopes that retained the pathogen-specific antigens whilst preserved the bacteria's native structure [17,70]. However, antigens on formalin killed bacterial cells might be masked [71] and could not stimulate strong immune responses. This probably was the reason that AVG elicited stronger systemic immune responses and protection than FKC.

In conclusion, our results provided new evidences to support that *A. veronii* ghosts (AVG) were generated by the expression of the PhiX174 lysis gene *E* and AVG could induce immune effects in koi. Not only did AVG stimulate the koi innate immune and specific immune system, but also those ghosts provided strong immune protection for koi, especially those infected by *A. veronii*.

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