Fish and Shellfish Immunology 84 (2019) 948–954

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Oral yeast-based DNA vaccine confers effective protection from *Aeromonas hydrophila* infection on *Carassius auratus*

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ARTICLE INFO

Keywords:
Oral administration
DNA vaccine
S. cerevisiae
A. hydrophila
Motile aeromonad septicemia
C. auratus

ABSTRACT

Our previous study has demonstrated that recombinant yeast can induce specific immune responses in *Carassius auratus* and may serve as a potential carrier for oral DNA vaccines in aquaculture. In this study, we further developed an effective yeast-based oral DNA vaccine against the bacteria *Aeromonas hydrophila*, which was expected to provide protection from the motile aeromonad septicemia (MAS). First, two candidate antigen genes, *ompG* and *omp48*, were cloned from the *Aeromonas hydrophila* genome DNA. Then, relative yeast-eukaryote shuttle vectors were constructed and their expression in eukaryotes was validated. Next, crucian carps were orally administered with *ompG* or *omp48* recombinant yeast, and the expression of the genes in the intestinal mucosa was confirmed by immunohistochemistry (IHC). The specific immune responses were further detected by Western blot and enzyme-linked immunosorbent assay (ELISA). The ELISA results showed that the production of the OVA-specific antibody in the *OVA-ompG* group was significantly higher than that of the *OVA-omp48* group, indicating that the *OVA-ompG* group elicited obviously stronger immune response than *OVA-omp48*. Finally, the challenge experiment against *Aeromonas hydrophila* infection demonstrated decreased fish mortality rate after the oral administration of the *OVA-ompG* yeast vaccine. In conclusion, our work provided a framework for the further development of oral yeast-based fishery vaccines.

1. Introduction

The aquaculture industry, particularly in China, has grown rapidly over the past three decades, with the greatest annual growth rate of all primary production sectors [1]. However, bacterial diseases, especially the motile aeromonad septicaemia (MAS) caused by *Aeromonas hydrophila* (*A. hydrophila*), are increasingly prevalent in cultured fish, resulting in considerable economic losses to the Chinese cyprinid fish industry [2,3]. Commercial antibiotics are traditionally used in fish health management to prevent MAS outbreaks. But, bacterial antibiotic resistance has become a major concern for the aquaculture industry [4,5]. On the other hand, vaccination is an important prophylactic measure used to prevent such diseases [6]. Several types of fish vaccines against *A. hydrophila* infection are available, including attenuated (live), inactivated (dead), and subunit vaccines [7]. However, the biosafety and economic costs of these vaccines limit their utility.

Saccharomyces cerevisiae (*S. cerevisiae*), commonly known as baker's yeast, is a non-pathogenic yeast strain mainly used in beer and bread industries. Our previous studies have demonstrated that recombinant *S. cerevisiae* can deliver recombinant protein [8,9]and exogenous DNA [10] as vaccines, as well as functional shRNA [11] to the intestinal dendritic cells (DCs) in mice by the oral administration. This yeast-based recombinant protein vaccine was also proved to function in rabbit [12]. What's more, our recent study further demonstrated that recombinant yeast can induce specific immune responses in *Carassius auratus* and may serve as a potential carrier for oral DNA vaccines in aquaculture [13]. Encouraged by this, we further attempted to develop the yeast-based oral DNA vaccines against the bacteria *A. hydrophila* for preventing the MAS.

The outer membrane proteins of the gram-negative bacteria like *A. hydrophila* have previously been proposed as vaccine candidates, because they are highly immunogenic and play an essential role in bacterial adherence to host epithelial cells [14,15]. Some outer membrane proteins from *A. hydrophila* have been shown to be immunogenic in fish, including the gourami, Indian major carp and the gold fish [16,17]. We thus selected two outer membrane protein genes from *A.*

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https://doi.org/10.1016/j.fsi.2018.10.065
Received 17 July 2018; Received in revised form 16 October 2018; Accepted 24 October 2018
Available online 25 October 2018

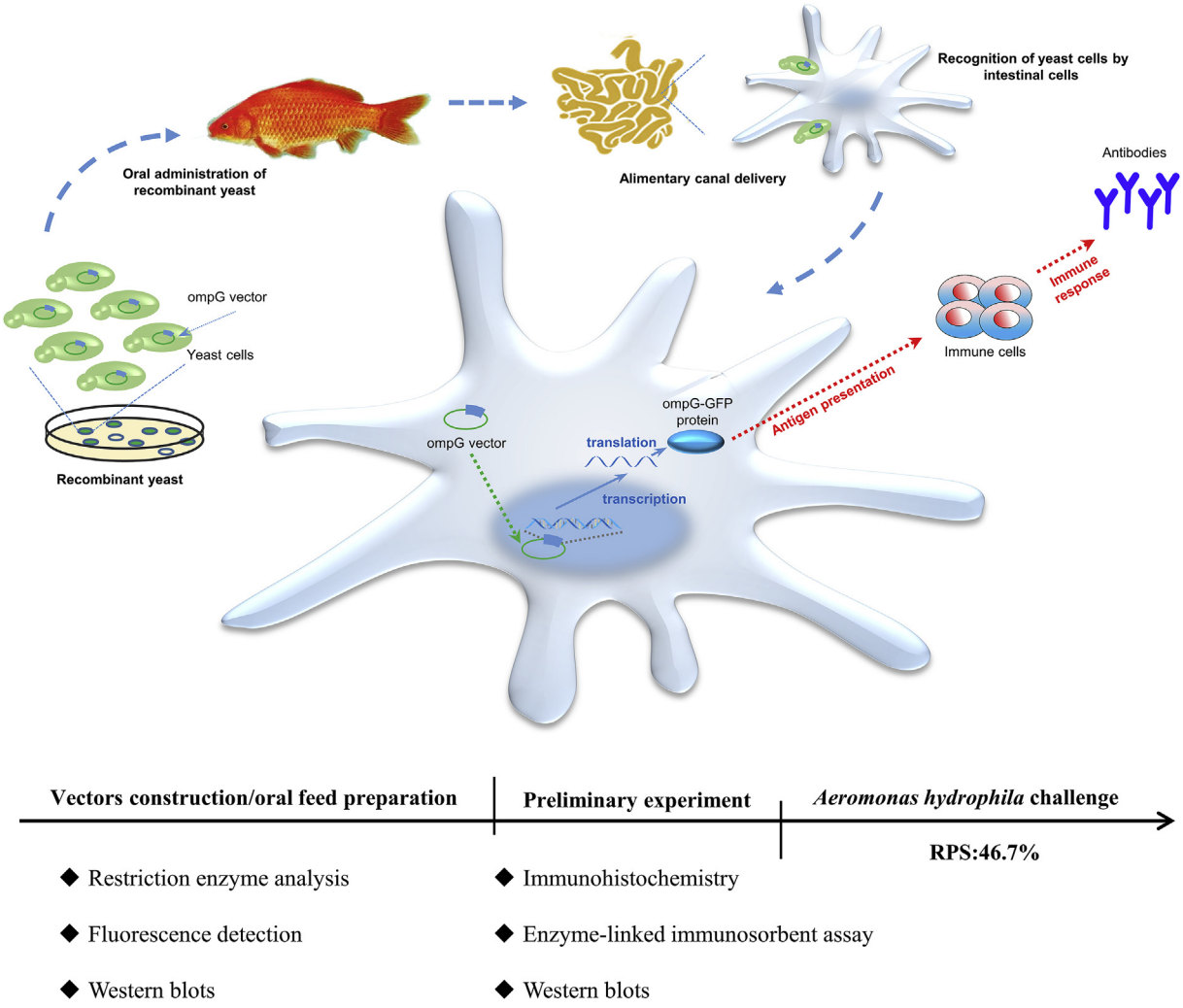

Fig. 1. The hypothetical mechanisms and concept map of the complete experiment, including vaccine preparation, the preliminary experiment, and the challenge experiment.

hydrophila (*ompG* and *omp48*) as target DNA antigens.

We aimed to deliver these genes into the intestinal mucosal system of the fish to induce an immune response against MAS [18,19] by orally administering the recombinant yeasts. The functional mechanism of the yeast-based oral vaccine, as well as the whole experiment design, including vaccine preparation, the preliminary experiment, and the challenge experiment, are shown in Fig. 1. We sought to develop an effective, inexpensive, orally-administered, yeast-based DNA vaccine to efficiently and conveniently prevent *A. hydrophila* infection in this study.

2. Materials and methods

2.1. Vector construction

The *ompG* gene (GenBank: CP016392.1) and *omp48* gene (GenBank: CP016392.1) were cloned from *A. hydrophila* genomic DNA with the primers OMP-GF/OMP-GR [16]and OMP48F/OMP48R [18]. PCR fragments and the JMB84-CMV-*OVA* vector (maintained in our lab) were digested with *Bam*HI/*Xho*I enzymes (NEB, UK). After gel extraction, these DNA products were ligated with T4 DNA ligase to construct the vectors JMB84-CMV-*ompG* and JMB84-CMV-*omp48*. Since OVA antigen promotes and enhances antigenicity, we used the primers Malt-F/OMP48R and OMP-GOF/OMP-GR to amplify these two genes, using the vectors, JMB84-CMV-*ompG* and JMB84-CMV-*omp48* as templates. We then digested PCR fragments and the JMB84-CMV-*OVA* vector with *Nhe*I/*Xho*I enzymes (NEB, UK). After gel extraction, DNA products were ligated with T4 DNA ligase to generate the JMB84-CMV-*OVA-ompG* and JMB84-CMV-*OVA-omp48* fusion vectors. We then digested the four newly-constructed vectors (JMB84-CMV-*ompG*, JMB84-CMV-*omp48,* JMB84-CMV-*OVA-ompG,* and JMB84-CMV-*OVA-omp48*) and the pET32a-HA-*OVA* vector (maintained in our lab) with *Bam*HI/*Xho*I enzymes (NEB, UK). The digested vectors were ligated to form pET32a-*ompG*, pET32a-*OVA-ompG*, pET32a-*omp48* and pET32a-*OVA-omp48* vectors.

To easily detect the specific antigen presenting in intestinal mucosal system *in vivo*, the primers GFP-F2/GFP-R and GFP-F1/GFP-R were used to attach a *T2A-GFP* cassette to the constructed vectors to form the fusion vectors: JMB84-CMV-*OVA-ompG-T2A-GFP* and JMB84-CMV-*OVA-omp48-T2A-GFP*, based on the pB-CMV-DsRed-CAG-NHEJ.Puro-*T2A-GFP* vector (maintained in our lab) (Fig. 2A). The procedures above were repeated using the *T2A-GFP* cassette, *Kpn*I/*Xho*I digestion, and T4 DNA ligase ligation. The accuracy of all the vectors was confirmed with enzyme digestion and sequencing. All of the primers used for vectors construction are given in Table S1.

2.2. Testing the successful expression of the target vector in eukaryotic cells

We used two cell lines to test whether the constructed vectors were expressed correctly in eukaryotic cells: the *Ctenopharyngodon idella*

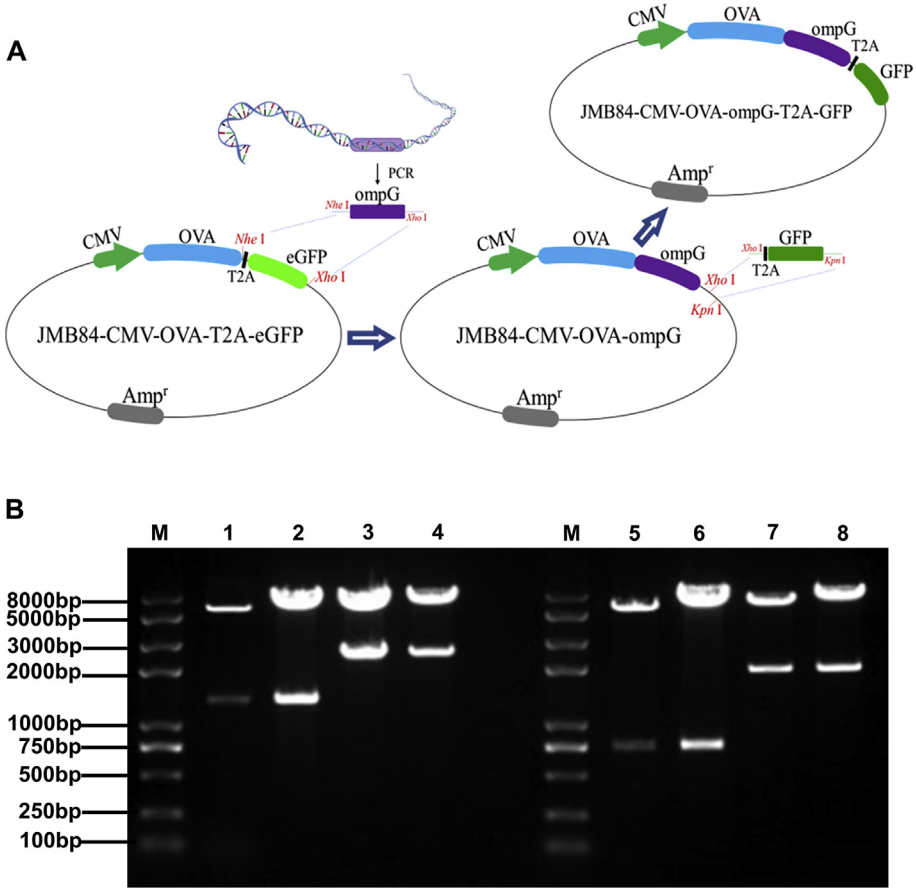

Fig. 2. Vector construction and validation. 2A, vector construction methodology. 2B, validation of the recombinant plasmid: lane M, DNA 2K PlusII DNA marker (TransGen Biotech, China); lane 1, pET32a-*omp48* vector; lane 2, JMB84-CMV-*omp48* vector; lane 3, JMB84-CMV-*OVA-omp48* vector; lane 4, JMB84-CMV-*OVA-omp48*-T2A-GFP vector; lane 5, pET32a-*ompG* vector; lane 6, JMB84-CMV-*ompG* vector; lane 7, JMB84-CMV-*OVA-ompG* vector; lane 8, JMB84-CMV-*OVA-ompG*-T2A-GFP vector. All the vectors were digested with *Xho*I and *Bam*HI. The *omp48* fragment was 1347 bp; the *OVA-omp48* fragment was 2542 bp; the *ompG* fragment was 749 bp; and the *OVA-ompG* fragment was 1938 bp.

kidney (CIK) cell line (provided by China Center for Type Culture Collection) and the HEK293T cell (maintained in our lab).

The CIK cell line was grown under 5% CO_2 at 28 °C in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (Biosource, USA), 100 U/ml penicillin (Sigma, USA), and 100 U/ml streptomycin (Sigma, USA), following a previous study [20]. When the cells reached 70–90% confluence, they were transfected with 500 ng JMB84-CMV-*OVA-ompG*-T2A-GFP and 500 ng JMB84-CMV-*OVA-omp48*-T2A-GFP using Lipofectamine 2000 (Invitrogen, USA) following the manufacturer's instructions. Cells were observed with a fluorescence microscope (Olympus, Japan) 48 h after transfection.

The HEK293T cell was grown under standard conditions at 37 °C under 5% CO_2. When the cells reached 70–90% confluence, they were transfected with six plasmids (JMB84 (maintained in our lab), JMB84-CMV-*OVA*, JMB84-CMV-*OVA-ompG*, JMB84-CMV-*OVA-ompG*-T2A-GFP, JMB84-CMV-*OVA-omp48*, and JMB84-CMV-*OVA-omp48*-T2A-GFP) using Lipofectamine 2000 (Invitrogen, USA), following the manufacturer's instructions. Cells were observed with a fluorescence microscope 48 h after transfection. At 72 h after transfection, proteins were collected from the cells using the RIPA lysis buffer (Beyotime, China). We used Western blot to detect the protein expression of target antigens, following a previous study [11]. Since the vectors carried the HA-tag, samples were first incubated with the primary antibody (anti-HA Tag mouse monoclonal antibody; 1:1000; Abbkine, USA) at 4 °C overnight. Samples were then incubated with the secondary antibody (goat anti-mouse Ig-HRP-conjugated; 1:2000; Sigma, USA) for 1 h before exposure.

2.3. Prokaryotic expression of antigen proteins

Plasmid vectors pET32a-*ompG*, pET32a-*OVA-ompG*, pET32a-*omp48*, and pET32a-*OVA-omp48* were transformed into *Escherichia coli* strain Rosseta (DE3). After cells reached an optical density at 600 nm (OD_{600}) of 0.6, antigen production was induced with 1 mM of isopropyl-thio-β-D-galactoside (IPTG) at 37 °C for 2 h with shaking. Next, bacterial cells were harvested by centrifugation at 12,000 g for 3 min. The cell pellets were then re-suspended in phosphate buffered saline (PBS), and the suspension was subjected to sonication. The inclusion bodies were then collected by centrifugation at 12,000 g for 15 min. We used polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot to confirm successful antigen expression following a previous study [10]. Samples where the target antigens were successfully expressed were incubated with the primary antibody (anti-His Tag mouse monoclonal antibody; 1:1000; Abbkine, USA) at 4 °C overnight, followed by a 1 h incubation with the secondary antibody (goat anti-mouse Ig-HRP-conjugated; 1:2000; Sigma, USA) before exposure.

2.4. Preparation of recombinant yeasts as oral feed

The eukaryotic expression vectors (including JMB84, JMB84-CMV-*OVA-ompG*, JMB84-CMV-*OVA-ompG*-T2A-GFP, JMB84-CMV-*OVA-omp48*, and JMB84-CMV-*OVA-omp48*-T2A-GFP) were transformed into JMY31 yeast (*MATa, ade2-1; ura3-1; his3-11; trp1-1; leu2-3112; can1-100*) as previously described [21], Single colonies were then selected and grown in a selection medium lacking uracil until OD_{600} was 1. Yeast cells carrying the JMB84 vector were cultured as controls. After being harvested and re-suspended in PBS, cells were heat-killed at 56 °C for 1 h [12]. The oral feed was prepared by thoroughly mixing suspended yeast cells basal meal powder, to yield 1.5×10^8 heat-killed yeast cells/g powder. This mixture was made into pellets using pellet former. Pellets were air-dried at room temperature and stored at −20 °C.

2.5. Experimental fish and oral vaccination

We used a preliminary experiment to determine the superior vaccine candidates. In the preliminary experiment, healthy crucian carp (*Carassius auratus*) (about 6 g body weight each, n = 5) were purchased from an aquarium market in Shaanxi, China. Fish were handled according to the policy and regulations for the care and use of laboratory animals. Fish were fed a proprietary pellet diet containing different recombinant yeasts as described previously [13], and were maintained at an appropriate temperature (~26–28 °C). Fish were allocated to one of four oral immunization regimens (n = 5 per group): PBS (control), yeast carrying JMB84 vector (control), yeast carrying JMB84-CMV-*OVA-ompG*-T2A-GFP (vaccination), or yeast carrying JMB84-CMV-*OVA-omp48*-T2A-GFP (vaccination). All the fish were fed a pellet diet equivalent to 3% of the total group body weight daily for 4 weeks.

2.6. Immunofluorescence

After five days of oral administration, two fish from the four treatment groups were anesthetized, and the second segment of the intestine was removed from each fish [23,24]. Intestinal samples were processed following a previously published protocol [25]. Paraffin-embedded tissue samples were cut into 5 μm sections with a microtome (Leica RM2235; Nussloch, Germany). Sections were incubated with the primary antibody (anti-GFP Tag mouse monoclonal antibody; 1:500; Abbkine, USA) at 4 °C overnight, then with the secondary antibody (goat anti-mouse Ig-FITC-conjugated; 1:100; Sigma, USA). Finally, stained slides and sections were mounted using Vecatshield mounting media (Vector, Burlingame, USA) containing Hoechst 33342 (Beyotime, China). Mounted slides and sections were observed under a confocal laser scanning microscope (A1R/A1; Nikon, Japan).

2.7. Antiserum preparation and detection of specific antibodies

After 28 days, serum samples were collected from the tail veins of three fish per treatment group, as previously described [26]. Mixed sera samples were used for Western blot, as previously reported [13]. The ompG/omp48 proteins induced above were used as antigens. We used the fish serum as the primary antibody, anti-carp (*Carassius spp.*) IgM monoclonal antibody (Aquatic Diagnostics Ltd, UK) as the secondary antibody, and HRP-conjugated goat-anti-mouse (Sigma, USA) as the tertiary antibody. Bands were viewed under a Leica DM2500M (Leica Microsystems GmbH; Wetzlar, Germany).

We used an enzyme-linked immunosorbent assay (ELISA) to determine the superior antigen candidate, as described previously [13]. We used eight dilutions (1:5, 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, and 1:640) to compare the antibody titers of the different treatment groups. A commercial OVA protein (Sigma, USA) was used as the antigen. ELISAs were performed following the manufacturer's instructions for the use of the anti-carp IgM monoclonal antibody (Aquatic Diagnostics Ltd, UK). Each antiserum dilution was assayed with three repeats.

2.8. A. hydrophila challenge

We constructed the challenge experiments based on the results of our preliminary experiment. 90 healthy crucian carp (*C. auratus;* about 6 g body weight each) were purchased from an aquarium market in Shaanxi, China. Then they were handled according to the policy and regulations for the care and use of laboratory animals. Fish were fed a proprietary pellet diet containing different recombinant yeasts, as described previously [13], and were maintained at an appropriate temperature (~26–28 °C). Fish were allocated to one of three oral immunization regimens (n = 30 per group): PBS (control), yeast carrying the JMB84 vector (control), and yeast carrying the JMB84-CMV-*OVA-ompG* vector (vaccine). At 28 days post-vaccination, fish were intraperitoneally injected with 30 μl of *A. hydrophila* suspension (5.0×10^6 CFU/mL), following a previous study [27]. Fish mortality was recorded daily, and dead fish were removed from each tank daily. The relative percent survival (RPS) was calculated following the method of Amend [28].

2.9. Statistical analysis

One-way analyses of variance (ANOVAs) were used to analyze all data and to identify differences among treatments, followed by an unpaired, two-tailed *t*-test. All the statistical analyses were performed using GraphPad Prism v5.01 (GraphPad, USA). The *P* value less than 0.05 and less than 0.01 were considered statistically significant and extremely significant, respectively.

3. Results

3.1. Vector and antigen expression verification

The *A. hydrophila* genes *ompG* and *omp48* were firstly cloned into the prokaryotic expression vector pET32a, and then a series of yeast-eukaryote shuttle vectors were constructed (Fig. 2A). All these constructs were confirmed by restriction enzyme analysis (Fig. 2B) and DNA sequencing.

CIK and HEK293T cells were transfected with JMB84-CMV-*OVA-ompG*-T2A-G.

FP and JMB84-CMV-*OVA-omp48*-T2A-GFP vectors carrying the GFP tag. The successful expression of the target genes was preliminary witnessed by fluorescence observation (Fig. 3A). Then, HEK293T cells were transfected with different yeast-eukaryote shuttle vectors, and the expression of the antigen genes was further confirmed by Western blot using the HA tag fused (Fig. 3B). These results demonstrated that the vectors were constructed correctly and were ready for subsequent recombinant yeast preparation and the oral administration.

Prokaryotic expression of the *ompG* and *omp48* genes were conducted to produce the corresponding proteins, which were required as antigens during the Western blot experiment for assessing the fish antiserum. The SDS-PAGE electrophoresis results of the prokaryotic expression proteins were shown in Fig. S1A. The prokaryotic expression proteins were also confirmed by Western blot using the HA tag fused to them (Fig. S1B).

3.2. Detection of antigen expression in the hindgut of orally vaccinated crucian carps

After functional verification of the expression vectors, recombinant yeast cells harboring JMB84-CMV-*OVA-ompG*-T2A-GFP and JMB84-CMV-*omp48*-T2A-GFP vectors were orally administrated to the fish. The expression of the GFP gene in the fish hindgut was detected by immunohistochemistry (IHC) five days after the first oral vaccine administration (Fig. 3C). The results suggested that the given DNA cassettes had been successfully delivered into intestinal mucosa of the crucian carps, and that the antigens could be expressed in fish intestinal cells, driven by the CMV promoter.

3.3. Detection of specific serum antibodies using western blot

4 weeks after the Crucian carps administered with the recombinant yeasts, the antiserums were sampled and subjected to Western blot analysis with prokaryotic expression ompG or omp48 proteins as antigens. The results demonstrated clear representative bands compared to the PBS and JMB84 controls (Fig. 3C), suggesting anti-ompG and anti-omp48 antibodies did exist in the serums and specific immune responses had been initiated in the orally yeast-vaccinated crucian carps.

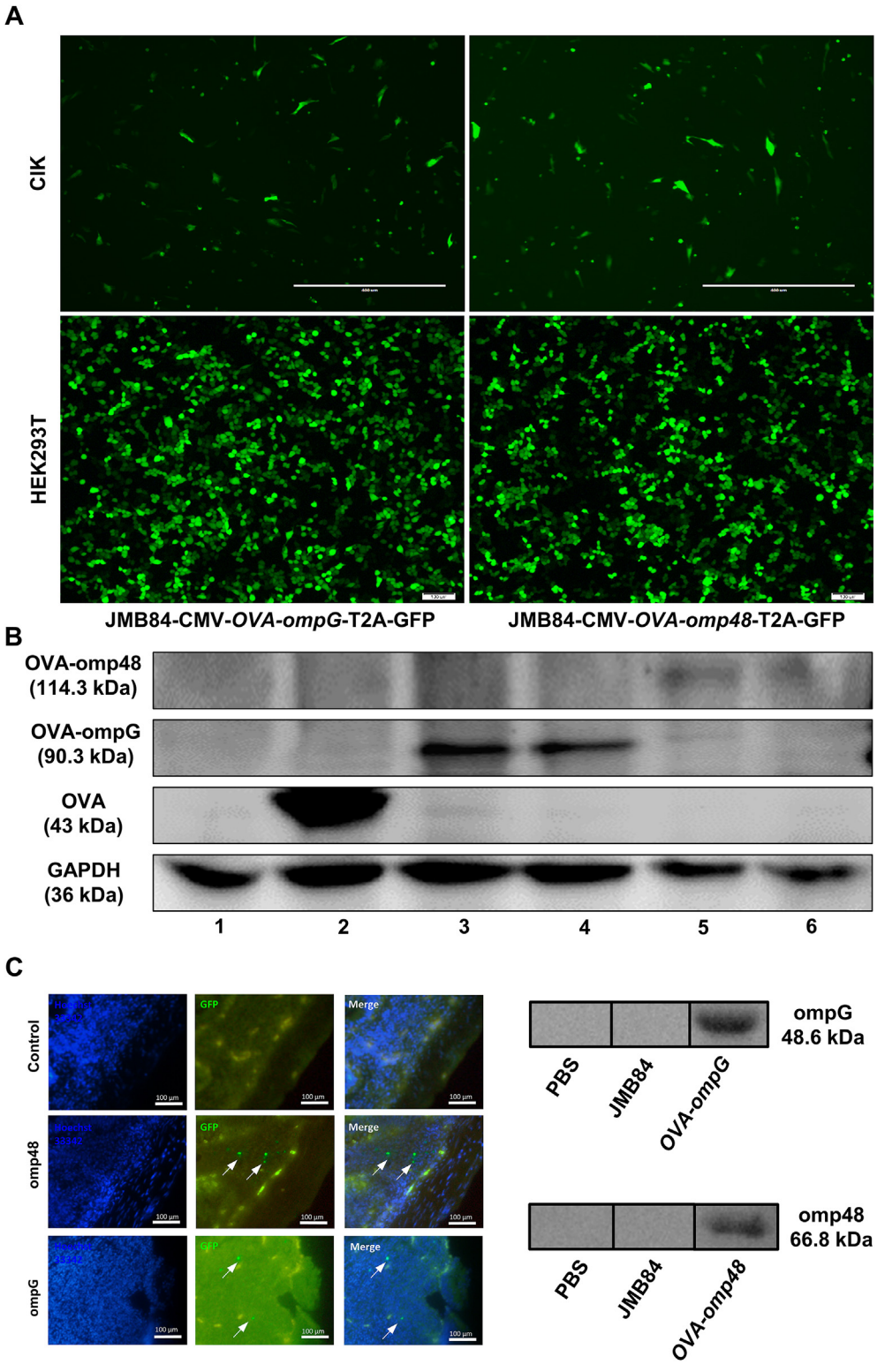

Fig. 3. The detection of the successful expression of the target vectors in eukaryotic cells; the successful presentation of DNA into the carp intestine; and the initiation of the specific immune response. 3A, the expression levels of the two vectors in two cells: *Ctenopharyngodon idella* kidney cells and HEK293T cells; 3B, protein expression levels in HEK293T cells transfected with different vectors as detected by Western blot. Lane 1, JMB84 vector; lane 2, JMB84-CMV-*OVA* vector; lane 3, JMB84-CMV-*OVA-ompG* vector; lane 4, JMB84-CMV-*OVA-ompG*-T2A-GFP vector; lane 5, JMB84-CMV-*OVA-omp48* vector; lane 6, JMB84-CMV-*OVA-omp48*-T2A-GFP vector; 3C, left panel, immunohistochemical detection of GFP expression in the carp hindgut after oral administration of the vaccine. DAPI was used for nuclear staining and FITC-conjugated goat anti-mouse IgG (H + L) was used to label GFP; right panel, detection of specific serum antibodies in the control and vaccinated groups with Western blot. The molecular weight of the ompG protein was 48.6 KDa and that of the omp48 protein was 66.8 KDa.

3.4. Identification of the superior vaccine candidate with ELISA

The titers for the antiserums from both orally yeast-vaccinated groups were further measured by ELISA. In all of the dilutions, the specific immune responses of the *OVA-ompG* group were stronger than that of the *OVA-omp48* group (Fig. 4A). We therefore selected the *OVA-ompG* recombinant yeast as the superior vaccine candidate for the subsequent challenge experiment.

3.5. Determination of vaccine protective efficacy

The *A. hydrophila* challenge results indicated that fish administered with our novel yeast vaccine were effectively protected against *A. hydrophila* infection (Fig. 4B). After challenge with *A. hydrophila* strain XS91-4-1, the RPS rate of the experimental group orally vaccinated with the *OVA-ompG* recombinant yeast was 46.7% (Table S2). Typical clinical symptoms of haemorrhagic septicaemia were observed in the dead fish. These results indicated that our vaccine conferred effective

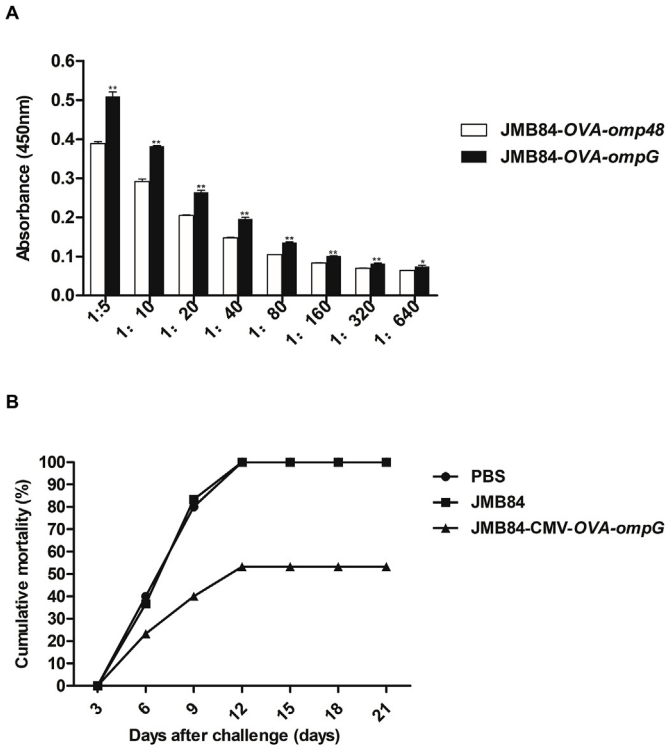

Fig. 4. Cumulative mortality of vaccinated fish, and the identification of the superior vaccine candidate using ELISAs. 4A, the specific antibody levels of the fish vaccinated with different DNA vaccines were determined using ELISAs. The values shown are the means of three assays ± SE. "**": $P < 0.01$; "*": $P < 0.05$; 4B, the accumulated mortality was calculated at the end of the monitoring period.

protection from *A. hydrophila* infection on the crucian carps.

4. Discussion

Currently, DNA vaccines (delivered intramuscularly) are one of the most promising types of vaccines targeting fish diseases [27]. However, these vaccines have several drawbacks: they stress the fish, they are labour and time intensive, they are expensive, and they may have safety issues. To overcome these obstacles, in this study, we developed an oral, yeast-based DNA vaccine that effectively prevented *A. hydrophila* infection on the crucian carps. Compared with currently used DNA vaccines, our yeast-based vaccine has the following particular advantages: (I) yeast zymosan acts as an adjuvant, boosting the immune response [29,30]; (II) DNA can be effectively delivered into the intestinal mucosal system via the phagocytosis of intestinal cells [31]; (III) yeast is easy to cultivate and non-harmful, and recombinant yeast is more cost effective and safer [32]; (IV) oral administration is easier than the injections required for current vaccines; and (V) recombinant yeast survives well in the fish intestine microenvironment, possibly because yeast resists digestion and the harsh conditions of the fish digestive tract [33].

Recombinant yeast was non-harmful and shown to act as an efficient delivery vehicle, transporting functional DNA to mammalian antigen presenting cells (APCs), especially dendritic cells [31,34,35], which provides a solid foundation for our study. More importantly, proof-of-concept studies have shown that yeast is a convenient vehicle for the transport of the model antigens *OVA* and *GFP* into the intestine cells of the crucian carp [13]. Based on these previous studies, we conducted this study and verified our hypothesis (Fig. 1), which proved the feasibility of oral yeast-based DNA vaccine in protecting fish against *A. hydrophila* infection. Moreover, dendritic cells may initiate antigen-specific adaptive immune responses in mammals, and they are uniquely potent inducers of primary immune responses *in vitro* and *in vivo* [36,37]. Recently, DCs-like APCs have been identified in teleost [38–40], and the uptake of yeast cells was shown in the Atlantic salmon intestine [41]. But, compared with the mature research in mice, there still lacks enough evidences for proofing the specific yeast-related swallow by teleost APCs. Be consistent with a previous report [13], we detected moderate levels of GFP expression in the intestinal tissue (Fig. 3C), which suggested that yeast cells might be engulfed by the fish intestinal cells and further indicated that special APCs-like intestinal cells were present in crucian carp.

In this study, we chose two outer membrane proteins genes as antigen candidates (omp48 and ompG) [18]. After adequate confirmation of vectors accuracy and feasibility of recombinant yeast as effective vehicle delivering DNA antigens into intestinal cells, we also tested the production of antigens-specific antibodies (Fig. 3C) of orally immunized fish using Western blots, which indicated that the recombinant yeasts effectively induced specific immune responses from the intestinal mucosal immune system. More importantly, the specific serum antibody titers induced by yeast-delivered *OVA-ompG* DNA was obviously higher than those induced by the yeast-delivered *OVA-omp48* DNA (Fig. 4A). This indicated that the antigen *OVA-ompG* is a superior antigen candidate which more efficiently induced an immune response than the antigen *OVA-omp48*. We therefore selected the *OVA-ompG* group for the challenge experiment. The challenge experiment results suggested that the *OVA-ompG* DNA vaccine protected the crucian carp against *A. hydrophila* infection, with an RPS of 46.7% over 21 days. Nevertheless, the protective effect of our yeast-based vaccine is still lower than other reported nanometre materials-mediated DNA vaccines through intramuscular injection [27], may owing to the vaccinated methods, materials characters, and the phagocytosis efficiency of intestinal cells in teleost, which need more efforts to enhance the effective of oral DNA vaccine. On the other hand, unlike other DNA vaccines, in consideration of the plasmid replication in yeast, it was not easy to determine the exact plasmid concentration in this yeast-based DNA vaccine. This must be further investigated in future work.

In summary, through this study we developed an effective and inexpensive, orally-administered, yeast-based *OVA-ompG* DNA vaccine, which conferred effective protection against *A. hydrophila* in live fish. Our work provides a framework for the further development of vaccines applicable to the aquaculture industry.

Author contributions

Zhiying Zhang supervised the project; Kun Xu financially supported the project; Baoquan Han and Kun Xu designed the experiments, performed the most of work, wrote and revised the manuscript; Zhongtian Liu and Wei Ge also performed part of work; Simin Shao, Nana Yan and Xinyi Li contributed important reagents and were helpful to care fishes and collect data.

Conflicts of interest

The authors declare that they do not have any conflicts of interest.

Acknowledgements

We would like to thank Xiaohui Chen and Yuwan Liu for their help with the related experiments. We are grateful to Dr. Samantha Johnson and LetPub (www.letpub.com) for the linguistic assistance. This research was supported by grants from the Fundamental Research Funds for the Central Universities of China (The Ph.D. Start-up Fund of Northwest A&F University, 2452015351).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://

doi.org/10.1016/j.fsi.2018.10.065.

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