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Protection against *Pseudomonas plecoglossicida* in *Epinephelus coioides* immunized with a *cspA1*-knock-down live attenuated vaccine

Weilu Qi[a,c], Wei Xu[b,1], Lingmin Zhao[a,c], Xiaojin Xu[a], Zhuhua Luo[b], Lixing Huang[a,c,*], Qingpi Yan[a,**]

[a] Fisheries College, Key Laboratory of Healthy Mariculture for the East China Sea, Ministry of Agriculture, Jimei University, Xiamen, Fujian, PR China
[b] Key Laboratory of Marine Biogenetic Resources, Third Institute of Oceanography, Ministry of Natural Resources, Xiamen, 361005, PR China
[c] Fujian Province Key Laboratory of Special Aquatic Formula Feed (Fujian Tianma Science and Technology Group Co., Ltd.), PR China

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ABSTRACT

Pseudomonas plecoglossicida is well-known as the cause of viscera granulomas disease in fish. In this study, a *cspA1* knock-down strain was constructed and tested in *Epinephelus coioides* to observe the changes in virulence and evaluate its potential as an attenuated live vaccine. The results showed that the *cspA1* knock-down strain caused a significant reduction in the ability of biofilm formation, motility, adhesion and virulence. *E. coioides* vaccinated with *cspA1* knock-down strain were more tolerant of the infection by wild-type *P. plecoglossicida*. The relative percent survival value of *E. coioides* vaccinated with *cspA1* knock-down strain reached 80% after challenging with wild-type *P. plecoglossicida*. In the meanwhile, the expression level of genes associated with immunity, including IL-1β, IgM, MHC-I and MHC-II, was up-regulated after vaccination, indicating that the *cspA1* knock-down strain can induce effective and durable immune response in *E. coioides* and it may be an effective attenuated live vaccine candidate for the prevention of infections by *P. plecoglossicida*.

1. Introduction

Pseudomonas plecoglossicida is an opportunistic Gram-negative pathogen. To date, infection of ayu [1], large yellow croaker (*Pseudosciaena crocea*) [2–4], orange spotted grouper (*Epinephelus coioides*) [5,6], and rainbow trout (*Oncorhynchus mykiss*) [7] with *P. plecoglossicida* has been reported. Outbreaks of *P. plecoglossicida* infection in cage-farmed *P. crocea*, which is characterized by white nodules in the internal organs of infected fish (including kidney, spleen and liver) and causes high mortality, have led to severe economic losses in the Fujian and Zhejiang provinces of China [8]. Outbreaks of *P. plecoglossicida* infection are mainly recorded in the seawater temperature range from 15 to 20 °C [9]. Our previous research with sequencing the transcriptome of *P. plecoglossicida* incubated under different temperatures (18 and 28 °C) further proved that changes of temperature can induce the regulation of its virulence, and showed that *cspA1*, a cold-shock protein (CSP) encoding gene was significantly high expressed under 18 °C [10].

Bacteria respond to the decrease of culture temperature in a specific manner. A temperature downshift results in inhibition of cell growth and proliferation, and changes in protein expression patterns [11]. The synthesis of most cellular proteins is inhibited after a decrease in culture temperature [12]. However, a number of proteins are found to be induced under cold-shock conditions, which are named as CSPs [11]. Expression of the CSPs reaches a maximum level during the phase of the cold-shock adaptation, the so-called acclimation. After this, the synthesis of CSPs decreased, establishing a new homeostasis level of lower protein expression than before cold shock [13]. It appears that CSPs not only play a major role during cold-shock adaptation but also are important under normal growth conditions [13]. Despite the fact that CSPs were extensively studied during the last decade, their exact function both at normal and cold-shock conditions is not elucidated yet.

Our previous results of RNA-seq [10] indicated that *cspA1* probably play an important role in the pathogenesis of *P. plecoglossicida*, but the specific mechanism is not clear. However, no research on function of CSPs in *P. plecoglossicida* has been reported. For better understanding of the function of *cspA1* in *P. plecoglossicida*, we constructed a *cspA1* silence strain via gene knock-down, then investigated the physiology and pathogenicity of the *cspA1*-RNAi strain. Moreover, we evaluated the immunoprotective potential of *cspA1*-RNAi strain, and revealed that

* Corresponding author. Fisheries College, Jimei University, Yindou Street 43, Xiamen, Fujian, 361021, PR China
** Corresponding author. Fisheries College, Jimei University, Yindou Street 43, Xiamen, Fujian, 361021, PR China
E-mail addresses: lixinghuang@outlook.com (L. Huang), yanqp@jmu.edu.cn (Q. Yan).
[1] Co-first author.

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Table 1
Primers for qRT-PCR.

Gene	Primers
cspA1	F:5′-ACAATGCGGGACGGTGAA-3′ R:5′-CAACTCCTTGAATGGCTCT-3′
IL-1β	F:5′-AGCCACAGTTCTGCCCTCAA-3′ R:5′-CCAACCTCATCATCGCCAC-3′
MHC-I	F:5′- GCCGCCACGCTACAGGTTTCTA-3′ R:5′- TCCATCGTGGTTGGGGATGATC -3′
MHC-II	F:5′-GGAGCCTCAGCCCAGCTTCA-3′ R:5′-CCAGTGGGAGGTCCTTCATG-3′
IgM	F:5′-TACAGCCTCTGGATTAGACATTAG-3′ R:5′-CTGCTGTCTGCTGTTGTCTGTGGAG-3′
CD4-1	F:5′-GTCTGAACGTTAGAGGGCTTTACA-3′ R:5′-CTGACAAACTCTGCGATTCCTAA-3′
CD8α	F:5′-AAGGACTTGGAGGATGACTTTAGGA-3′ R:5′-TTCCTGAACCTCCTACTGAAATCCT-3′
CD8β	F:5′-CCCGTCAAGGTTCTTTATCC-3′ R:5′-CATTGGTGATTCGTAGCGTAA-3′
16s rDNA	F:5′-GTTGGGAGGAAGGGCAGTAAG-3′ R:5′-ATCTAGGCATTTCACCGCTACA-3′
β-actin	F:5′-ACGCTCTGCCTCACGCCATC-3′ R:5′-GGTGGTGAAGCTGTAGCC-3′

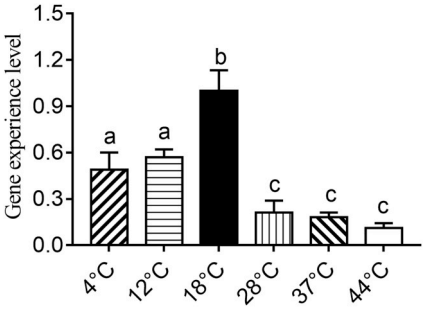

Fig. 1. qRT-PCR analysis of the expression of *cspA1* under different temperatures. The data are presented as the means ± S.D. (n = 6). The means of the treatments not sharing a common letter are significantly different at $P < 0.05$.

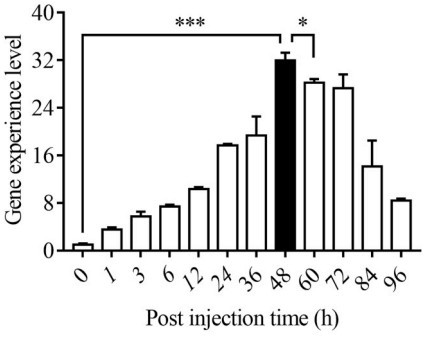

Fig. 2. qRT-PCR analysis of the expression level of *cspA1* in the wild type *P. plecoglossicida* infected *E. coioides* spleen at different stages of infection. Data are presented as mean ± S.D. (n = 3). *$P < 0.05$, ***$P < 0.001$.

cspA1 possess the potential of being a target to construct live attenuated vaccine to combat *P. plecoglossicida* in *E. coioides*.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The pathogenic *P. plecoglossicida* strain (NZBD9) was isolated from the spleen of naturally infected large yellow croaker with white-spot disease [14]. The NZBD9 strain was routinely grown in LB (Luria Bertani) medium at 4, 12, 18, 28, 37 or 44 °C with shaking at 220 rpm. *E. coli* DH5α was obtained from TransGen Biotech (Beijing, China), which was grown in LB medium (37 °C, 220 rpm).

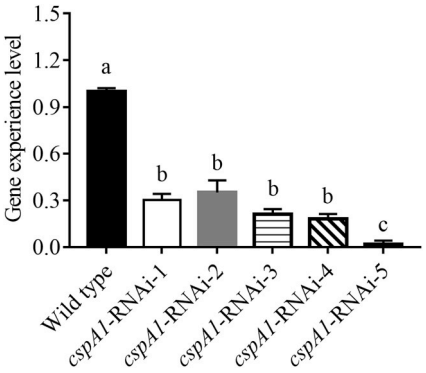

Fig. 3. Construction of *cspA1*-RNAi strains of *P. plecoglossicida*. The *cspA1* expression levels of 5 *cspA1*-RNAi silence strains are shown as means ± SD from three independent biological replicates. Means of treatments not sharing a common letter are significantly different at $P < 0.05$.

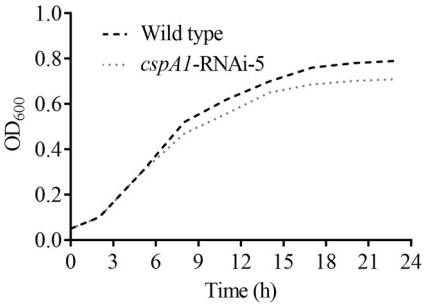

Fig. 4. Growth curve of wild-type strain and *cspA1*-RNAi strain.

2.2. Construction of cspA1 knock down strain

RNAi strain was constructed according to methods described before [15]. Five short hairpin RNA sequences targeting *cspA1* were designed respectively and synthetized by Shanghai Generay Biotech Co., Ltd. (Shanghai, China) (Table S1). After linearizing pCM130/tac vectors (which include resistance to tetracycline) with the restriction enzymes *Nsi*I and *Bsr*GI (New England Biolabs, U.S.A), the oligonucleotides were annealed and ligated to the linearized pCM130/tac vectors using T4 DNA ligase (New England Biolabs) following the manufacturer's recommendations. The recombinant pCM130/tac vectors were transformed into the competent *E. coli* DH5α cells by heat shock and then were extracted and electroporated into *P. plecoglossicida*. Finally, the expression level of *cspA1* of each RNAi strain was detected by quantitative real-time PCR (qRT-PCR) as we described before [16].

2.3. Characterization of the silencing strain of cspA1

The *cspA1* silencing strain was characterized by biofilm formation, motility, *in vitro* adhesion, and growth. The biofilm assay for *P. plecoglossicida* was performed using the crystal violet stain method as described by Huang et al. [17], and then quantitated by measuring OD_{570} nm, six independent biological replicates were performed for each data point. For the assay of motility of *P. plecoglossicida* strains, the soft agar method was adapted [5], while the diameters of the colonies were measured at 24 h (n = 6). *In vitro* adhesion was measured according to a procedure reported by Kong et al. [17], while the stained adherent bacteria cells were counted by microscopy (× 1000) (n = 6). The growth of *P. plecoglossicida* strains were detected as previous description [5].

Table 2
Characteristics of *cspA1*-RNAi-5 strain.

Characteristics	Wild type	*cspA1*-RNAi-5
Biofilm formation (OD_{570})	1.11 ± 0.24	0.27 ± 0.06**
Motility (mm)	7.6 ± 0.12	1.7 ± 0.13**
Adhesion (cells per field of view)	1440 ± 36	120 ± 16**

2.4. Artificial infection and sampling

All animal experiments were carried out strictly under the recommendations in the 'Guide for the Care and Use of Laboratory Animals' set by the National Institutes of Health. The animal protocols were approved by the Animal Ethics Committee of Jimei University (Acceptance NO JMULAC201159).

Three hundred and fifty healthy *E. coioides* (average weight 17.9 ± 1.4 g) were obtained from Zhangzhou (Fujian, China) and were acclimatized at 18 °C for one week under specific pathogen-free laboratory conditions. Fish were tested to be healthy by sera agglutination and bacteriological recovery tests as described by Pang et al. (2018) [18]. For virulence comparison through survival assays, twenty *E. coioides* were injected intraperitoneally with 100 μL 10^4-10^9 cfu/mL wild-type or *cspA1*-RNAi-5 strain suspended in sterile phosphate buffered saline (PBS). *E. coioides* that were intrapleurally injected with PBS were used as a negative control. The fish were monitored for 14 days at 18 ± 1 °C, and infected fish were observed for morbidity and mortality daily.

For detection of *cspA1* during the infection process, the spleens of six weight-matched *E. coioides* infected with 100 μL 1.8×10^5 cfu/mL (10^3 cfu/g) wild type strain *P. plecoglossicida* were sampled at each time point including 1, 3, 6, 12, 24, 36, 48, 60, 72, 84, 96 hpi.

2.5. RNA isolation

TRIzol reagent (Invitrogen, USA) was used for total RNA extraction from bacterial cells or *E. coioides* spleens, as directed by the manufacturer. Reverse transcription was carried out with A Reverse Aid Mu-MLV cDNA synthesis kit (TransGen Biotech, China) from 2.0 mg total RNA, as instructed by the manufacturer [17].

2.6. qRT-PCR

qRT-PCR was carried out using a QuantStudio 6 Flex real-time PCR system (Life Technologies, U.S.A). All primer sequences are provided in Table 1. The expression of bacterial genes was normalized using *16s rRNA*. In *E. coioides*, the expression of genes was normalized to *β-actin*. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative level of gene expression.

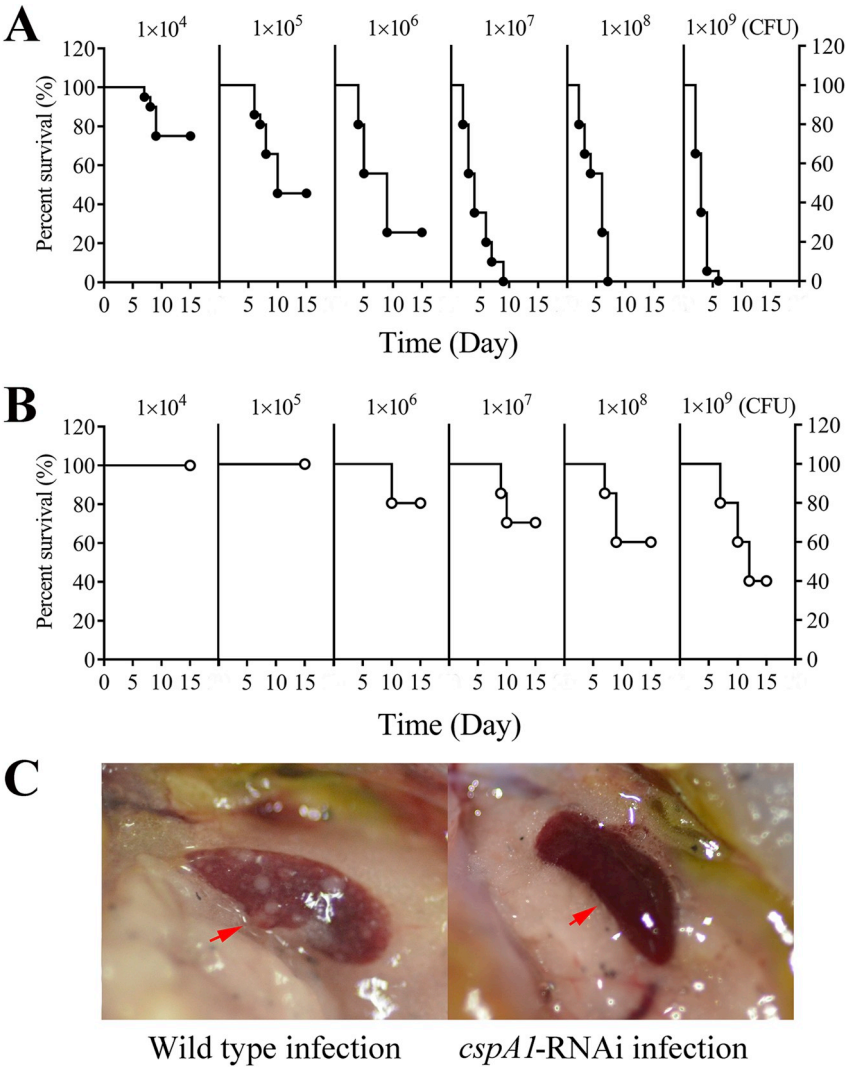

Fig. 5. The virulence comparison of wild-type and *cspA1*-RNAi strain of *P. plecoglossicida*. **(A–B)** Survival curves of *E. coioides* challenged with *P. plecoglossicida* wild-type **(A)** and *cspA1*-RNAi-5 **(B)**. **(C)** Symptoms of spleen of *E. coioides* infected by *P. plecoglossicida* wild-type and *cspA1*-RNAi-5.

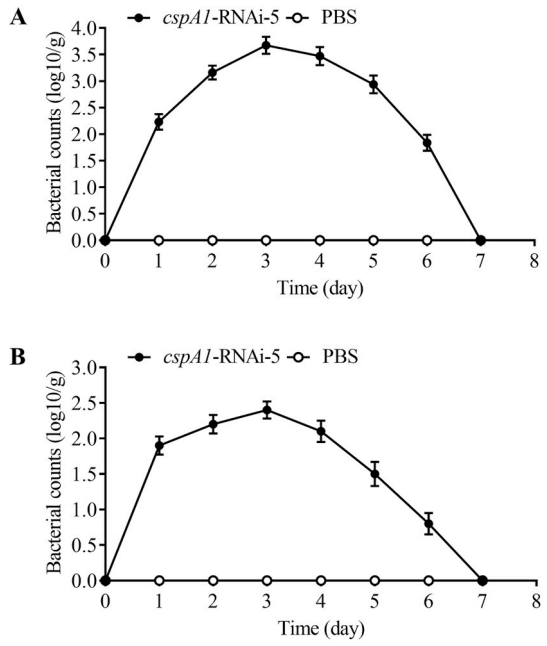

Fig. 6. Propagation of *cspA1*-RNAi-5 in *E. coioides* spleen **(A)** and head-kidney **(B)** after injection. Control fish were injected with equal volume of sterile PBS. The number of viable bacteria was shown as the mean ± standard of three samples.

2.7. E. coioides vaccination

E. coioides (average weight 17.9 ± 1.4 g) were randomly divided into two groups and intraperitoneally injected with 100 μL 10^5 cfu/mL *cspA1*-RNAi-5 strain or equal volume of PBS. All the fish were maintained at 18 °C. *cspA1*-RNAi-5 strain infected *E. coioides* spleens were sampled at 2, 5, 7, 14, 21 and 28 day after vaccination for qRT-PCR analysis of the expression of immune-related genes. Three *cspA1*-RNAi-5 and PBS injected *E. coioides* spleens and head-kidneys were aseptically collected from day 1 to day 7. All the samples were weighed and homogenized in 1 mL PBS. The homogenates were serially diluted and plated in triplicate onto LB plates (10 μg/mL Tetracycline) and incubated at 28 °C for 18 h. The bacteria counts were calculated by dividing the weights of the tissues and from the mean of three samples.

Four weeks after vaccination, 30 *E. coioides* per group were intraperitoneally injected with 100 μL 1×10^8 cfu/mL of wild-type strain. The water temperature during infection was 18 ± 1 °C. The daily mortality of infected *E. coioides* was recorded.

2.8. Statistical analysis

Data were presented as mean ± standard deviation (SD), and were assessed with SPSS 18.0. Differences were determined by analysis of one-way ANOVA followed by the Dunnett's multiple comparison test. $P < 0.05$ indicated statistical significance.

3. Results

3.1. Validation of cspA1 expression under different temperatures, and during the infection process

Expression levels of *cspA1* under 4 °C, 12 °C, 18 °C, 28 °C, 37 °C, 44 °C temperatures were assessed by qRT-PCR. The results showed that the expression level of *cspA1* under 18 °C was significantly higher than under the other temperatures (Fig. 1). Meanwhile, the expression of *cspA1* was higher at low temperatures than at high temperatures. In addition, we used qRT-PCR to detect the expression of *cspA1* gene with the progressing of infection. The expression level of *cspA1* increased with the progressing of infection and reached its peak at 48 h after infection, then gradually decreased (Fig. 2).

3.2. Construction of the cspA1-RNAi strain

Five *cspA1* stable silencing strains were constructed, and the expression level of *cspA1* was detected. The results showed that the expression level of *cspA1* in the silencing strains decreased significantly, indicating that the silencing strain was successfully constructed (Fig. 3). Among them, the *cspA1*-RNAi-5 strain exhibited the best efficiency of gene silencing, so it was selected as the object of further research.

3.3. The growth curve, biofilm formation, motility, adhesion and virulence of cspA1 silencing strain

The growth curves of wild-type and *cspA1*-RNAi-5 strain were measured respectively, which showed that, the growth rate of *cspA1*-RNAi-5 strain decreased slightly compared with the wild-type (Fig. 4). Then, comparison of biofilm formation between wild-type and *cspA1*-RNAi-5 strain showed that, the biofilm production of *cspA1*-RNAi-5 was 4.11 folds lower than that of the wild-type (Table 2). Meanwhile, *cspA1*-RNAi-5 strain showed a significantly decreased motility and adhesion ability compared with the wild-type (Table 2). Furthermore, the virulence of *cspA1*-RNAi-5 was significantly decreased (Fig. 5). No clinical symptoms or mortalities were detected within 2 weeks following challenge of the fish with doses less than 10^5 cfu/mL of *cspA1*-RNAi-5 strain. The LD_{50} of wild strain and *cspA1*-RNAi-5 were 7.4×10^4 and 2.2×10^8, respectively.

3.4. Investigation of the livability of cspA1-RNAi-5 strain in vivo

cspA1-RNAi-5 strain was able to disseminate into but survive transiently in fish spleen and head-kidney then was gradually eliminated from the host body (Fig. 6). The highest bacterial number was detected in spleen and head-kidney on day 3.

3.5. Immune gene expression in E. coioides after vaccination with cspA1-RNAi-5 strain and immune protective effects of cspA1-RNAi-5 strain in E. coioides

qRT-PCR was carried out to analyze the transcription levels of genes encoding *MHC-I*, *MHC-II*, *IgM*, *IL-1β*, *CD4-1*, *CD8α*, and *CD8β* in the spleen of *E. coioides*. The results showed that all 7 genes were significantly induced by *cspA1-RNAi-5* strain (Fig. 7).

The immune protection effect of *cspA1-RNAi-5* was evaluated by injecting wild-type *P. plecoglossicida* into *E. coioides* which survived after injected with PBS and *cspA1-RNAi-5* strain, respectively. The results indicated that most of the *E. coioides* with *cspA1-RNAi-5* inoculation survived after injection with *P. plecoglossicida* wild-type, and the immune protection rate was as high as 80%, whereas mortality in the group administered PBS was 100% (Fig. 8).

These results suggested that *cspA1-RNAi-5* can induce the immune response of *E. coioides*, so as to play an immune protection role on the host against *P. plecoglossicida*.

4. Discussion

In this study, the stable silencing strain was constructed and *E. coioides* was artificially infected. The results showed that, compared with wild-type, the virulence of the stable silencing strain of *cspA1* was significantly reduced. In addition, our results showed that *cspA1* expression levels were extremely active in the course of infection of wild-type. These results indicated that *cspA1* could be involved in virulence regulation and infection process of *P. plecoglossicida*. Considering that CSP is a kind of temperature sensor, while *cspA1* is highly expressed

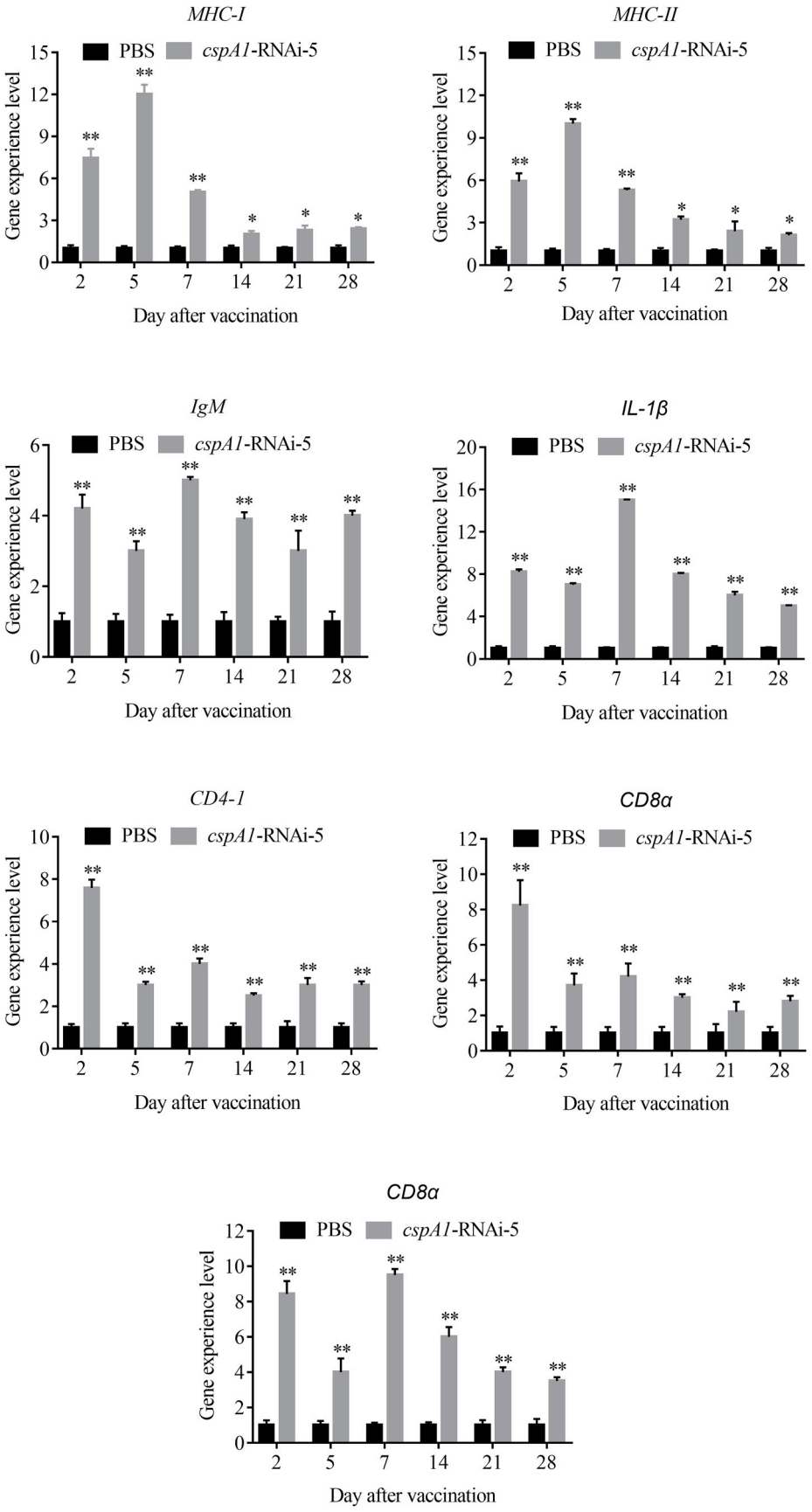

Fig. 7. qRT-PCR analysis of the expression of immune-related genes in *cspA1*-RNAi-5 strain infected *E. coioides* spleen at 2, 5, 7, 14, 21 and 28 day after vaccination. The data are presented as the means ± S.D. (n = 6). The means of the treatments not sharing a common letter are significantly different at $P < 0.05$.

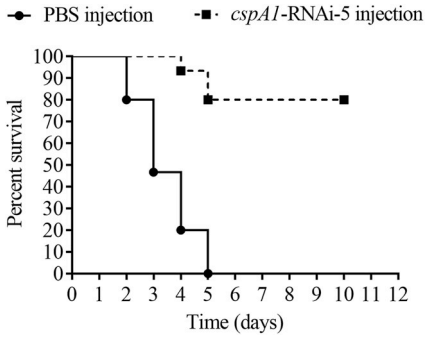

Fig. 8. Survival in *E. coioides* vaccinated with *cspA1*-RNAi-5 strain and PBS following challenge with wide-type *P. plecoglossicida*.

specifically at the pathogenic temperature of the temperature dependent pathogenic *P. plecoglossicida*, *cspA1* may play an important role in temperature related virulence regulation of *P. plecoglossicida*.

CSPs are nucleic acid binding proteins regulating the expression of various genes including those involved in stress resistance and virulence in bacteria [11–13]. For example, CSPs are involved in virulence, cell aggregation and flagella-based extracellular motility of *Listeria monocytogenes*. A *L. monocytogenes* mutant deleted in *csp* is attenuated with respect to human macrophage infection as well as virulence in a zebrafish infection model. Moreover, this mutant is incapable of aggregation and fails to express surface flagella or exhibit swarming motility [19]. Phenotypic assays *in vitro* also demonstrated a crucial role for these proteins in membrane stress, motility, and biofilm formation in *Salmonella* [20]. Our results showed that there were significant differences between wild-type and *cspA1*-RNAi-5 strain in motility, adhesion and biofilm production. Therefore, our results revealed that *cspA1* contributed in multistep of *P. plecoglossicida* pathogenesis, which was consistent with reports on the virulence regulation by CSPs in other pathogenic bacteria.

Compared with different kinds of chemical drugs, developing and applying vaccines to prevent major aquatic diseases is becoming an effective alternative for aquaculture industry. Because the vaccine not only improves the specific immunity of animal body, but it can also meet the requirements of non-polluting environment and non-drug-residue in aquatic products [21]. Meanwhile, live attenuated vaccines is found to induce a more robust cell-mediated and humoral immune response than inactivated vaccine [22]. In this study, we evaluated the possibility of *cspA1* as a potential target for constructing *P. plecoglossicida* live attenuated vaccine by knocking out *cspA1* in *P. plecoglossicida*.

MHC I and *II* are found in the jawed vertebrates which can display antigen from within the cell to cytotoxic T cells and thus trigger an immediate response of the immune system [23]. *IgM* is the largest antibody, which appears firstly in the response to antigen exposure [24]. *IL-1β*, the first interleukin to be characterized, is crucial to the initiation and regulation of immune and inflammatory responses in many economically important teleost [25]. CD4 and CD8 are two subsets of T lymphocytes and play important roles in the recognition and presentation of antigens in specific immune responses. Furthermore, via dual RNA-seq, *MHC-I*, *MHC-II*, *IgM*, *IL-1β*, *CD4-1*, *CD8α*, and *CD8β* were proved to play key roles in the host-pathogen interaction during *P. plecoglossicida* infection of *E. coioides* [6]. In this study, the elevated expression of these immune-related genes (*MHC-I*, *MHC-II*, *IgM*, *IL-1β*, *CD4-1*, *CD8α*, and *CD8β*) confirmed that *cspA1*-RNAi-5 strain can stimulate the host to produce immune protection. In addition, we evaluated the efficacy of *cspA1*-RNAi-5 as a live attenuated vaccine by injection route in an *E. coioides* model, resulting in a RPS of 80% post vaccination.

Taken together, with gene silencing, we revealed that *cspA1* gene was involved in the multistep pathogenesis of *P. plecoglossicida*. At the same time, the silencing strain exhibited a considerable protection effect against *P. plecoglossicida* challenge, and could elicit both cell mediated and humoral immune responses in *E. coioides*. These results further proved the importance of *cspA1* in *P. plecoglossicida* and provided a reference for further study on this virulence factor.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.04.029.

Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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