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A T3SS Regulator Mutant HY9901 Δ*araC* of *Vibrio alginolyticus* Decreased the Expression of HopPmaJ and Provided Protection to *Danio rerio* as a Live-Attenuated Vaccine

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Vibrio alginolyticus, a zoonotic bacterial pathogen, expresses a type III secretion system (T3SS) that is critical for pathogen virulence and disease development. In this study, the mutant HY9901 Δ*araC* was obtained from the laboratory and its biological characteristics were analyzed. The swimming ability of Δ*araC* decreased and exhibited a 2,600 times reduction in virulence to zebrafish. However, Δ*araC* showed no difference in growth and extracellular protease activity compared to wild type. Biofilm-forming ability was improved at 24 h, but no difference was observed at other time points. The results of drug sensitivity testing showed that compared with the wild-type HY9901 strain, Δ*araC* was sensitive to amikacin, tetracycline, neomycin, minocycline, and gentamicin. The transcription levels of T3SS effector proteins HopPmaJ, VopS, VcrV, and VopN were analyzed by qRT-PCR. The results showed that Δ*araC* had significantly upregulated the mRNA expression of VopS, VcrV, and VopN, but significantly downregulated the mRNA expression of HopPmaJ at each stage compared with HY9901. Western blotting and the β-galactosidase reporter gene experiment also showed that the deletion of *araC* gene significantly downregulated the expression of HopPmaJ. Finally, the relative percent survival (RPS) rate of grouper inoculated by intramuscular (IM) injection of HY9901 Δ*araC* was 61.3% after being challenged with HY9901. Real-time qPCR analysis showed that vaccination of HY9901 Δ*araC* could enhance the expression of immune-related genes, including *gata-1*, *il6*, *IgM*, *il-1β*, and *lyz* in liver and spleen, indicating that Δ*araC* applied as a live-attenuated vaccine effectively induced an immune response in the zebrafish. This study provides evidence for the subsequent development of an effective live-attenuated *V. alginolyticus* vaccine.

Keywords: *Vibrio alginolyticus*, type III secretory system, gene *araC*, characteristics, live-attenuated vaccine

INTRODUCTION

Vibrio alginolyticus exhibits characteristics of a halophilic and mesophilic Gram-negative bacterium (Rameshkumar et al., 2014; Yang et al., 2021). It is commonly present in the microfauna of the marine environment and a variety of marine animals. It is one of the main pathogens of fish, shrimp, shellfish, and other mariculture animals (Bunpa et al., 2020). In addition to *V. alginolyticus* representing a conditional pathogen of fish, it poses a serious threat to human public health and threatens the Chinese aquaculture industry, with reports of it causing human food poisoning, otitis media, septicemia, and other diseases (Li et al., 2009; Bunpa et al., 2018). Due to the large-scale outbreak of *V. alginolyticus* and the long-term overuse and abuse of a large number of antibiotics and other chemical drugs, drug resistance of *Vibrio* is increasing daily with major implications to the aquaculture industry (Shahimi et al., 2021).

The mechanism of bacterial drug resistance is complex (Stewart, 2001), and there are limited reports on the pathogenic mechanisms of *V. alginolyticus*. So far, researchers have not solved this problem. Prophylactic prevention of disease outbreaks caused by *V. alginolyticus* through vaccination is a key objective for marine aquaculture, to minimize impact to the water environment and human health while improving the survival rate of aquatic animals (Aly et al., 2021). Therefore, understanding key molecules with roles in virulence can enable identification of potential vaccine candidates and can also avoid the bacterial drug resistance problem, which continues to plague the aquaculture industry.

Research on the pathogenic mechanisms of different bacteria have revealed that many Gram-negative bacteria contain many broadly conserved genes, which encode a type III protein secretion system (Hueck, 1998). When *V. alginolyticus* contacts and infects the host, the Type III secretion system (T3SS) operates through the injection of bacterial effector proteins into the host cells, resulting in the death of those cells (Zhou et al., 2013). In recent years, research relating to the *V. alginolyticus* T3SS has elucidated the role of T3SS apparatus proteins (Zhou et al., 2013; Pang et al., 2022), chaperone proteins (Wu et al., 2020), effector proteins (Pang et al., 2018), and regulatory proteins (Liu et al., 2016; Zhou et al., 2020). From current knowledge on *Vibrio parahaemolyticus* and *Pseudomonas aeruginosa*, the transcription of *V. alginolyticus* T3SS genes is regulated by ExsA of the AraC/XyIS family of transcription regulators (Yahr and Wolfgang, 2006; Liu and Thomas, 2015; Liu et al., 2016).

In addition to activating the expression of virulence-related genes, AraC family members can also activate the expression of carbon metabolism and stress response genes (Yang et al., 2013). AraC is an AraC family transcriptional regulator, However, the function of *V. alginolyticus* AraC is poorly understood. In *Escherichia coli*, AraC is active in the form of a dimer and reacts with small effector molecules bound to the N-terminal domain of protein, so as to regulate carbon metabolism (Lowden et al., 2010). Gene *araC* has the ability to positively regulate L-arabinose metabolism because it can promote the expression of L-arabinose structural genes *araA*, *araB*, and *araD*, and *araBAD* encodes three metabolic enzymes required for L-arabinose metabolism (Sheppard and Englesberg, 1967; Singer and Englesberg, 1971). Moreover, at the molecular level, the details of AraC protein response to arabinose have been clarified (Schleif, 2000; Yang et al., 2011). HopPmaJ is a known effector protein of *V. alginolyticus* eliciting pathogenic effect on orange-spotted grouper (*Epinephelus coioides*). Diseased fish showed symptoms of ulcer, liver, and kidney bleeding and swelling (Pang et al., 2018). Moreover, regulatory protein TyeA can upregulate the expression of HopPmaJ (Wu et al., 2020); thus, in order to further understand the function of AraC in T3SS, we cloned the *V. alginolyticus araC* gene and analyzed it bioinformatically (MO et al., 2021). We obtained the mutant strain HY9901 Δ*araC* and studied its biological characteristics and pathogenicity, and showed that AraC regulates the transcription of T3SS effector proteins. In addition, here we report on the potential of HY9901 Δ*araC* mutant as a candidate live-attenuated vaccine against *V. alginolyticus* in zebrafish and the associated proinflammatory and immunoglobulin-related immune response induced.

MATERIALS AND METHODS

Bacterial Strains, Plasmid, and Experimental Fish

The bacterial strains, plasmids, and zebrafish used in this study are listed in **Table 1**. V. *alginolyticus* HY9901 was isolated from diseased red snapper (*Lutjanus sanguineus*) (Cai et al., 2007) in Zhanjiang Port, Guangdong Province. Healthy zebrafish (*Danio rerio*) were purchased from Zhanjiang Aquatic Market. The average length and weight were 3 cm and 0.3 g, respectively. The zebrafish were tested by bacteriological recovery tests and kept in seawater in a circulation system at 28°C for 2 weeks prior to the experiment. The primers were synthesized by Sangon Biotech Co., Ltd.

Construction of In-Frame Deletion Mutant of *araC* Gene

In-frame deletion was conducted according to the method used previously (Liu et al., 2016). The upstream and downstream homologous arms of *araC* coding sequence were amplified. Except for *Bgl*II and *Sal*I restriction site sequences, the two amplified fragments contained 10-bp overlapping sequences. These products were used as templates for PCR, and the fusion fragments were amplified by primers at both ends of the upstream and downstream homologous arms. The whole fragment was digested with *Bgl*II and *Sal*I and connected to the suicide vector pDM4 (enzyme digestion). The obtained plasmid was electroporated into *E. coli* S17-1 and transferred into *V. alginolyticus* HY9901 by electroporation. Ampicillin and chloramphenicol were used to screen the monoclonal strains successfully transferred into plasmids on TSA plates. Several insertional mutant single colonies were taken, scribed on an LB plate (4 g/L L-arabinose), and cultured at 28°C for 18 h. Colonies on the plate were selected. Single colonies with the

TABLE 1 | Bacterial strains, plasmids, and experimental fish used in this study.

Strains, plasmids	Relevant characteristics	Source
V. alginolyticus HY9901	Wild type	(Cai et al., 2007)
pDM4	A suicide vector with ori R6K sacB; Cmr	(Liu et al., 2016)
Δ*araC*	*araC* deletion mutant	This study
pME6522	pVS1-p15A *E. coli–Pseudomonas* shuttle vector for transcriptional lacZ fusions and promoter probing, Tcr	This study
pME6522-*hop–lacZ*	Promoter sequences of hop cloned into pME6522	This study
E. coli S17-1 (λpir)	*thi pro hsdR hsdM+ recA* RP4-2-Tc::Mu-Km::Tn7λpir	(Simon et al., 1983)
S17-*hop–lacZ*	S17 carrying pME6522-*hop–lacZ*	This study
hop–lacZ:: Δ*araC*	Promoter sequences of *hop* was inserted in the upstream of lacZ gene in Δ*araC* strain	This study
hop–lacZ:: HY9901	Promoter sequences of *hop* was inserted in the upstream of lacZ gene in HY9901 strain	This study
Zebrafish	Experimental fish, purchased from Zhanjiang Aquatic Market	Zhanjiang Aquatic Market

primer araC-up/araC-down were detected using the wild-type *V. alginolyticus* as the control. After single-colony purification, amplification verification was carried out again, and the sequencing results were verified to determine whether the mutant strain Δ*araC* was successfully constructed.

Characterization of the Δ*araC* Mutant *Vibrio alginolyticus*

Genetic Stability Testing of Mutant HY9901 *ΔaraC*

The insertion mutant Δ*araC* was continuously subcultured 30 times on TSB medium. The primers araC-F/araC-R were used to detect the genetic stability of the mutant at the 30th generation.

Growth Curve of Bacteria

Bacterial growth curves were performed according to Shi et al. (2021). Wild strain HY9901 and mutant strain Δ*araC* (OD_{600} = 0.5) were inoculated at a ratio of 1:100 in fresh TSB medium and cultured at 28°C; OD_{600} was measured every 2 h including 3 replicates per group. After taking the average value, a growth curve was drawn.

Swarming Motility

Bacterial swarming motility was performed according to the method of Pearson (Pearson, 2019). A single colony of HY9901 and the mutant strain Δ*araC* were inserted vertically into the LBS (0.3% Agar and 2% NaCl added to the LB medium). The diameter of the swimming circle was measured after 12 h during incubation at 28°C.

Detection of Extracellular Protease Activity

Extracellular protease activity was assessed according to the method of Zhang et al. (2022) with modifications. HY9901 and mutant strain Δ*araC* were coated on TSA plates with sterile cellophane, cultured at 28°C for 24 h, washed with PBS, and centrifuged at low temperature for 30 min; 0.22-μm porous membranes were used to filter the supernatant. Protease activity of the supernatant was measured using azocasein trichloroacetic acid colorimetric determination of solution at OD_{442}.

Detection of Biofilm Formation Ability Using Crystal Violet Ammonium Oxalate

The crystal violet staining method was performed according to O'Toole (2011). HY9901 and mutant strain Δ*araC* were cultured to OD_{600} = 0.5; 100 μl/well was inoculated onto sterile 96-well plates and cultured at 28°C for 6 h, 12 h, 24 h, 48 h, and 72 h. After 72 h, the bacterial solution was washed with PBS and dried; 150 μl of methanol was added for fixation and dried; 150 μl/well crystal violet ammonia oxalate solution was added, rinsed with water, and then dried upside down before the addition of 150 μl of ethanol. The absorbance was read at OD_{570}. The experiment was repeated three times.

Detection of Biofilm Formation Ability Using Laser Scanning Confocal Microscope (LSCM)

Biofilm formation was investigated using a laser scanning confocal microscope (LSCM) as described by Miquel Guennoc et al. (2017). HY9901 and Δ*araC* (OD_{600} = 0.5) were diluted 50-fold, added to glass-bottom culture dishes (spec: type 28.2 mm, class diameter: 20 mm) (Wuxi NEST, Wuxi, China), and statically cultured in a 28°C biochemical incubator for 24 h. Both strains were gently washed three times with PBS, and then combined and incubated with 10% SYTO9 green in the dark for 20 min, washed three times with saline, mounted in 40% saline-glycerol, and observed by confocal microscopy (Zeiss, LSM710, Germany). The excitation wavelength was 488 nm, scanned from the bottom to the top of the biofilm, Z-section was 1 μm apart, and biofilm parameters—biomass and maximum thickness—were determined. Three samples were assessed for each strain and the average readout was calculated.

Dose Response Challenge Test (LD_{50})

The injection concentrations of wild-type strain HY9901 and Δ*araC* were 10^4, 10^5, 10^6, 10^7, and 10^8 CFU/ml. The water temperature is controlled to 28°C. In the experimental group, each fish was injected with 5 μl of bacterial solution by intramuscular injection. The control group was treated with 5 μl of PBS (**Table 3**). The fish were observed for 14 consecutive days until the mortality was stabilized.

Antibiotic Susceptibility

Using the paper diffusion method (Cai et al., 2016), HY9901 and Δ*araC* (OD_{600} = 0.5) were coated on TSA plates, pasted with 30 kinds of antibiotic-sensitive paper, and cultured at 28°C for 12 h, and the sensitivity to the antibiotic was measured and evaluated with vernier caliper.

Transcriptome Sequencing

HY9901 and Δ*araC* were cultured in DMEM medium at 28°C until OD_{600} = 0.5, reinoculated into DMEM medium and cultured at 28°C for 12 h. The bacterial solution was centrifuged, and the precipitated cells were collected and dissolved in Trizol (Takara bio, Inc.). Novogene Co., Ltd. conducted a series of experiments, including mRNA extraction, RNA fragmentation, cDNA synthesis, and RNA SEQ library construction.

Expression Analysis of T3SS-Related Genes

RNA of HY9901 and Δ*araC* was provided by Novogene Co., Ltd. The T3SS effector genes in this study were *hop* (Pang et al., 2018), *vopN* (Tam et al., 2010), *vopS* (Osorio, 2018), and *vcrV* (Kinoshita et al., 2020). 16S rRNA is used as an internal reference. The primers are shown in **Table 2**. Based on the method of Li et al. (2016b), cDNA was synthesized and real-time PCR was used to analyze the expression of T3SS-related genes.

Preparation of Proteins and Western Blot Analysis

In order to detect the effect of AraC deletion on the secretion of effector protein HopPmaJ, we cultured wild strain HY9901 and deletion strain Δ*araC* for 12 h with DMEM. The culture was harvested, washed, and mixed evenly with PBS. A sample combination of 5:1 sample to 6× Protein Loading Buffer (#DL101-02, purchased from Trans Gen Biotechnology Co., Ltd.) was boiled and centrifuged to obtain the whole bacterial protein. After SDS-PAGE, proteins were electrophoretically transferred to a PVDF membrane. The membrane was blocked with TBST solution containing 5% skimmed milk and probed with HopPmaJ serum antibody (the prokaryotic expression vector pET28a-*hop* was constructed and HopPmaJ serum antibody was prepared by Sangon Biotech Co., Ltd., 1:5,000 diluted in TBST solution). The second antibody was HRP-labeled Goat Anti-Rabbit IgG (#A0208, purchased from Shanghai Beyotime Biotechnology Co., Ltd.) (1:8,000 diluted in TBST solution) and colored by ClarityTM Western ECL substrate (#1705061, purchased from Bio-Rad Co., Ltd.). The automatic chemiluminescence image analysis system (TAN 5200) was used to take photos and record the experimental result.

TABLE 2 | Sequences of primers used in this study.

Primer name	Primer sequence (5′-3′)	Accession number
IL-6-F	GGTCAGACTGAATCGGAGCG	NM_001079833.1
IL-6-R	CAGCCATGTGGCGAACG	
IL-6R-F	GCATGTGCTTAAAGTATCCTGGTC	NM_001114318.1
IL-6R-R	TGCAAATTGTGGTCGGTATCTC	
IL-1*β*-F	TGGACTTCGCAGCACAAAATG	AY340959.1
IL-1*β*-R	GTTCACTTCACGCTCTTGGATG	
IL-8-F	GTCGCTGCATTGAAACAGAA	XM_001342570.2
IL-8-R	CTTAACCCATGGAGCAGAGG	
IgM-F	GTTCCTGACCAGTGCAGAGA	AF246193
IgM-R	CCTGATCACCTCCAGCATAA	
gata-1-F	CCGTTGATGTAGATGAACC	BC164788.1
gata-1-R	CCAGAATTGACTGAGATGAG	
rag-1-F	GAAGTATACCAGAAGCCTAAT	NC_007136.7
rag-1-R	TTCCATTCATCCTCATCACA	
TLR5-F	GAAACATTCACCTGGCACA	NC_007131.7
TLR5-R	CTACAACCAGCACCACCAGAATG	
c/ebp*β*-F	GCCGTACCAGACTGCTCCGA	NC_007119.7
c/ebp*β*-R	AGCCGCTTCTTGCCTTTCCC	
β-actin-F	ATGGATGAGGAAATCGCTGCC	NM_131031.1
β-actin-R	CTCCCTGATGTCTGGGTCGTC	
Lyz-F	GGCAGTGGTGTTTTTGTGTC	NM_139180.1
Lyz-R	CGTAGTCCTTCCCCGTATCA	
hop-F	CTTCGCTTTCGGTTTGCT	KX245315
hop-R	AATACCATCCCACCCTGT	
vopS-F	AGTTTTGGAAGTGTTAGCG	ACY41053.1
vopS-R	ACATTGCCTCTGTCATCG	
vopN-F	TGAACTCGTTTCGGACTA	ACY41067.1
vopN-R	ACTTTCTGGACTCGCACT	
vcrV-R	CGACAGGAGCAACAGACA	MG907044.1
vcrV-F	CGTTTGTCCCGAGATAGTT	
16S-F	TTGCGAGAGTGAGCGAATCC	NR_044825.2
16S-R	ATGGTGTGACGGGCGGTGTG	
araC-F	ATGAAATCTAGCTTAAGCA	MN328349
araC-R	CTATCGGGAAAACTCTCTA	
araC-up	AACTTGTGGGCTAGAATGTGTCC	
araC-down	TAATGCCAATGACCCCGAT	
pME-hop-F	CCGGAATTCTCCGATAAACGTTAAACC	
pME-hop-R	GTTCTGCAGTATTCCTCTATCCCTACT	

Construction of Single-Copy lacZ Reporter Fusions and *β*-Galactosidase Assay

The recombinant plasmid of the pME6522-hop promoter region was constructed. A 363-bp DNA fragment was amplified upstream of hop by primer pME-hop-F/pME-hop-R. PCR products were digested with *Pst*I and *Eco*RI and cloned into plasmid pME6522 to construct pME6522-*hop-lacZ*. The plasmid was transformed into *E. coli* S17 to obtain strain S17-*hop-lacZ*, respectively, and integrated into *V. alginolyticus* HY9901 and mutant strain Δ*araC* through conjugation to obtain lacZ transcription fusion strains *hop-lacZ*::HY9901 and *hop-lacZ*::Δ*araC*. *hop-lacZ*::HY9901 and *hop-lacZ*::Δ*araC* (OD_{600} = 0.5) were inoculated into fresh TSB at a ratio of 1:100 and cultured for 18 h. After collection, the strains were applied separately to β-galactosidase detection kits (#RG0036, purchased from Shanghai Biyuntian Biotechnology Co., Ltd.).

TABLE 3 | Experiment of LD_{50}.

Concentration (CFU/ml)	HY9901	Mortality rate (%)	Δ*araC*	Mortality rate (%)	Control (PBS)	Mortality rate (%)
10^8	10 × 3	90	10 × 3	30	-	
10^7	10 × 3	80	10 × 3	20	-	
10^6	10 × 3	60	10 × 3	10	-	
10^5	10 × 3	20	10 × 3	0	-	
10^4	10 × 3	20	10 × 3	0	-	
0 (PBS)	–		–		10 × 3	0

This allowed assessment of β-Galactosidase activity to judge the regulatory relationship between AraC and effector protein HopPmaJ.

Safety Assessment

LD_{50} experiment confirmed that 10^5 CFU/ml Δ*araC* was not lethal to zebrafish (**Table 3**). The mutant Δ*araC* was cultured in TSB, and the concentration was adjusted to 10^5 CFU/ml with PBS and the water temperature was maintained at 28°C. Two groups (30 tails/group) were injected intramuscularly with 5 μl of Δ*araC* (10^5 CFU ml^{-1}), and PBS was used as negative control and injected in the same volume. Liver and spleen were sampled continuously for 7 days. The ground zebrafish viscera were diluted with PBS and coated on a TCBS plate. They were cultured at 28°C for 18 h and counted.

Vaccine and Challenge

Vaccination was undertaken according to Ding et al. (2019) with modifications. The water temperature was kept at 28°C. Two groups (80 tails/group) were injected intramuscularly with 5 μl of Δ*araC* (10^5 CFU ml^{-1}), and each fish in the control group was injected with 5 μl of PBS. Four weeks post vaccination, each group (n = 30) was randomly assigned and challenged by intramuscular injection of 5 μl of *V. alginolyticus* HY9901 (1×10^8 CFU ml^{-1}). Fish were monitored for 14 days. RBS was calculated in the light of the formula (RPS (%) = (1 − immunized group mortality/control group mortality) × 100%).

Histological Sections and Pathological Observation of Spleen and Liver of Zebrafish

Histological analysis was conducted to evaluate safety of HY9901Δ*araC* as an attenuated vaccine. After 28 days post-immunization, three zebrafish in the control group and the immunized group were randomly selected for sampling, and the spleen and liver tissues were dissected for histopathological observation. The negative control was a PBS-injected control group, and the positive control was the wild strain HY9901-injected group. Organ samples were fixed in 4% paraformaldehyde for 24 h and transferred to 70% ethanol. The samples were processed through an ethanol gradient, transferred to xylene, embedded in paraffin, and sectioned at 5 μm. The sections were sealed after hematoxylin–eosin (H.E.) staining, and observed and photographed under an optical microscope.

Immune Gene Expression of Zebrafish Induced by HY9901Δ*araC* Vaccine

After 4 weeks of immunization, the liver and spleen of zebrafish were collected, added with transzol, and homogenized. According to Li et al. (2016b), total RNA was prepared, and reverse transcription, real-time qPCR detected immune-related genes' expression levels. Primers for *il-6*, *il-6r*, *il-1β*, *il-8, igm*, *gata-1*, *lyz*, *rag-1*, *tlr5*, and *c/ebpβ* are shown in **Table 3**, and β-actin was used as internal reference.

Ethics Statement

All animal experiments were conducted strictly based on the recommendations in the *Guide for the Care and Use of Laboratory Animals* set by the National Institutes of Health. The animal experiments were approved by the Animal Ethics Committee of Guangdong Ocean University (Zhanjiang, China).

Biosecurity

The bacteria protocols were approved by the Biosecurity Committee of Guangdong Ocean University (Zhanjiang, China).

Statistical Analysis

The experimental data were analyzed by single-factor analysis of variance (ANOVA) with SPSS19.0 software. ** indicates highly significant difference compared with the control group ($p < 0.01$). * indicates significant difference compared with the control group ($p < 0.05$).

RESULTS

Construction and Verification of HY9901Δ*araC*

The 1,109-bp fragment was amplified by the correct deletion mutation clone, and the length of the HY9901 amplified fragment was 1,827 bp. After cloning and purification, it was amplified and verified again, and the PCR products were submitted for

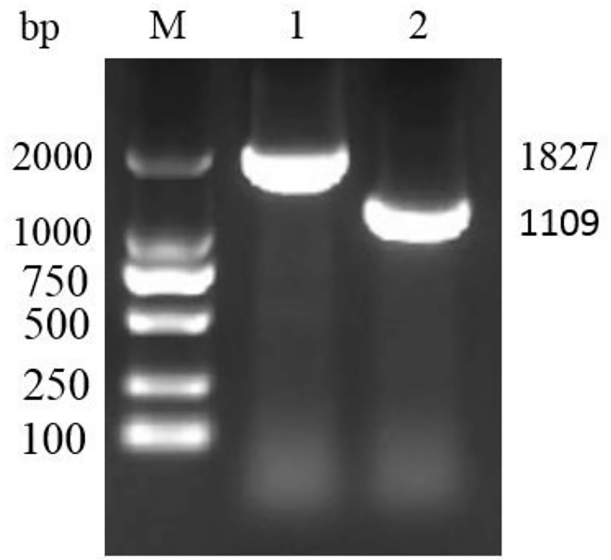

FIGURE 1 | Construction and confirmation of the knockout mutant strain HY9901ΔaraC. M: DL2000 marker; Lane 1, the 1,827-bp fragment amplified from genomic DNAs of the strain HY9901; Lane 2, the 1,107-bp fragment amplified from genomic DNAs of ΔaraC using primer pairs of araC-up/araC-down.

sequencing. The sequencing results confirmed that the mutant strain Δ*araC* was successfully constructed **Figure 1**.

Characterization of the *araC*

Genetic Stability of Mutants

After the mutant Δ*araC* was continuously subcultured for 30 generations, primers araC-F/araC-R were used in PCR to amplify mutant strain Δ*araC* and the wild strain. The amplified products were detected by 1.5% agarose gel electrophoresis. The results showed that for the wild strain HY9901, a fragment of 711 bp was obtained. The mutant strain Δ*araC* was negative for this fragment (**Figure 2A**), indicating that the deletion strain Δ*araC* can be inherited stably when the gene *araC* is deleted.

Comparison of Growth Rates of HY9901 and *ΔaraC*

The growth rate of HY9901 and Δ*araC* was similar, and the deletion of *araC* gene has no effect on the growth of *V. alginolyticus* ($p > 0.05$) (**Figure 2B**). The exponential growth phase of the two bacterial strains was from 0 to 6 h, with growth stationary at 18 h, $OD_{600} \approx 1.8$.

Swarming Motility

HY9901 and Δ*araC* were inoculated on the LBS, and the results were as follows: the swarming circle of HY9901 was 39.7 ± 0.4 mm, and that of Δ*araC* was 30.8 ± 0.5 mm (**Table 4**). The swarming circle diameter of HY9901Δ*araC* was significantly smaller than that of HY9901 ($p < 0.01$), indicating that the swarming ability of HY9901 Δ*araC* was significantly weakened.

Extracellular Protease Activity

Compared with wild strain HY9901, there was no significant difference in the extracellular protease activity of Δ*araC* ($p > 0.05$) (**Table 4**).

Detection of Biofilm Formation Ability

The biofilm thickness was detected by crystal violet staining (**Figure 2C**) and confocal scanning (**Figure 2D** and **Table 4**). Crystal violet results showed that there were no significant differences in the ability of wild strain HY9901 and mutant strain Δ*araC* to form biofilms before 24 h ($p > 0.05$), The ability of Δ*araC* to form a biofilm was stronger than that of wild strain ($p < 0.01$). There was little difference after 48 h. Confocal electron microscopy (24 h) (**Figure 2D**) 2.5D results showed that the biofilm of Δ*araC* was significantly thicker than that of the wild strain HY9901 ($p < 0.01$).

LD_{50} Determination

LD_{50} results showed that the 50% lethal dose of HY9901Δ*araC* was 3,620 times higher than that of the wild strain (**Tables 2**, **4**). The symptoms of body surface hyperemia, abdominal redness and swelling, and slow swimming were observed in the diseased fish. The results showed that the virulence of HY9901 Δ*araC* was significantly decreased when compared with the wild strain ($p < 0.01$).

Antibiotic Susceptibility

According to CLSI standards, the susceptibility of the mutant strain Δ*araC* and wild strain HY9901 to 30 kinds of antibiotics was determined by disk diffusion method. Compared with the wild strain, the sensitivity of the Δ*araC* strain to amikacin, tetracycline, and neomycin changed from resistant to moderate sensitivity; the sensitivity of the Δ*araC* strain to minocycline and gentamicin changed from drug resistant to high sensitivity; and the sensitivity of the Δ*araC* strain to chloramphenicol changed from moderate sensitivity to high sensitivity (**Table 5**).

TABLE 4 | Comparison of biological characteristics between HY9901 and HY9901Δ*araC*.

Characteristics	HY9901	HY9901Δ*araC*
Activity of ECPase (A_{422})[a]	1.01 ± 0.2	0.94 ± 0.2
Biofilm thickness (μm)[b]	120 ± 20	160 ± 20**
Swarming (mm)[c]	39.7 ± 0.4	30.8 ± 0.5**
LD_{50}(CFU ml^{-1})[d]	5.8×10^5	$2.1 \times 10^{9**}$

*Values are mean ± standard deviation for three trials. Significant differences between HY9901 and HY9901ΔaraC indicated by asterisk. **p < 0.01.*

[a]Bacteria were incubated in TSB for 18 h at 28°C.

[b]Bacteria were incubated in a glass bottom culture dish (NEST) for 24 h at 28°C.

[c]Swarming diameters were measured after 24-h incubation on LBS containing 0.3% agar and 2% NaCl.

[d]LD_{50}were evaluated in healthy zebrafish with an average weight of 0.3 g.

Transcriptome Sequencing Analysis of HY9901 and Strain Δ*araC*

Through the transcriptome sequencing analysis of HY9901 and mutant strain Δ*araC*, a total of 1,376 differentially expressed genes were screened. Compared with HY9901, 635 genes were upregulated and 741 genes were downregulated in Δ*araC* (**Table S1**). A total of 429 differentially expressed genes were enriched by GO function. The results showed that the differentially expressed genes of mutant strain Δ*araC* and HY9901 were mainly related to locomotion, cilium, or flagellum-dependent cell motility and bacterial-type flagellum (**Figure 3**).

T3SS-Related Gene Expression Analysis

qRT-PCR was employed to analyze the transcription levels of T3SS-related effector genes at each time point (6, 12, 24, 48, and 72 h). The results showed that compared with wild strain HY9901, Δ*araC* significantly decreased the expression of *hopPmaJ* (*hop*) (6, 12, 24, 48, and 72 h) ($p < 0.01$), but increased

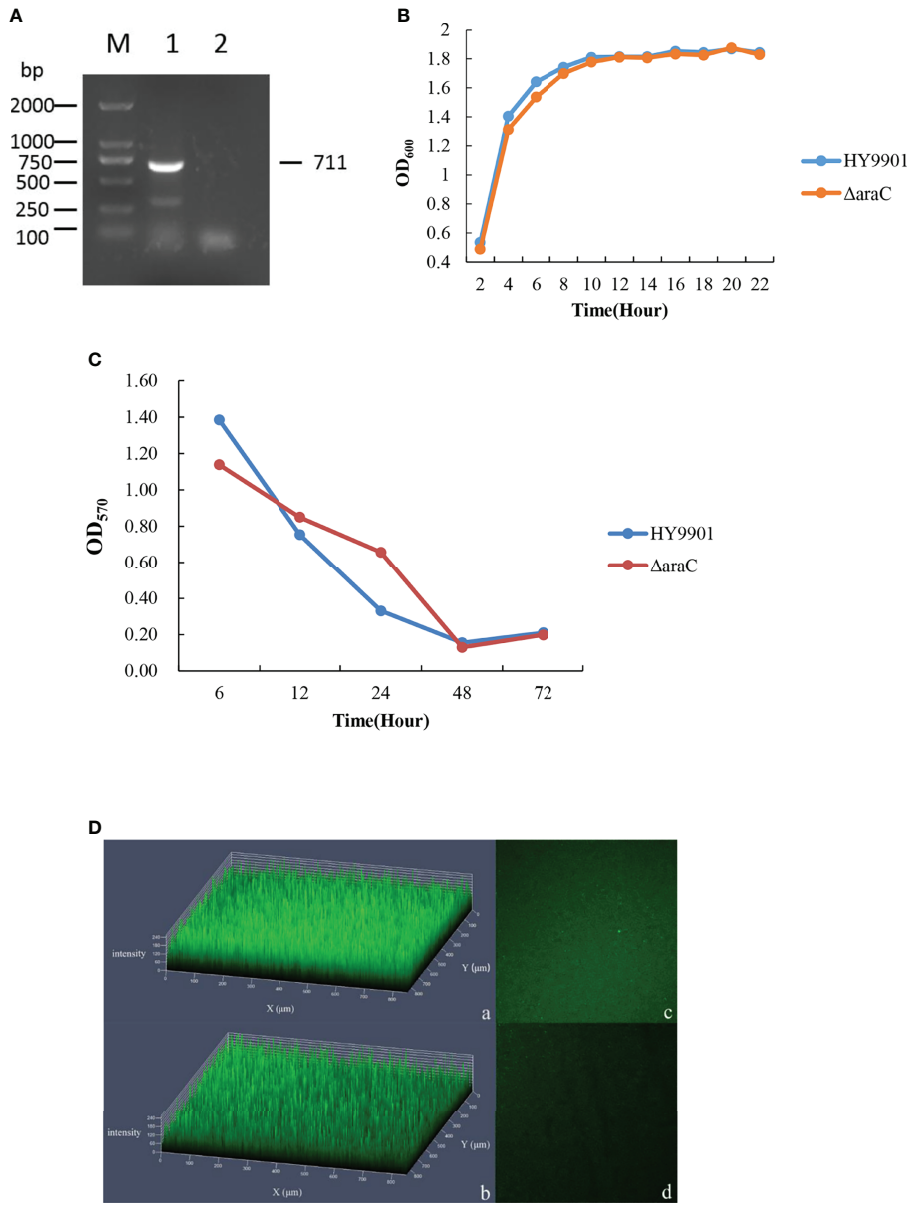

FIGURE 2 | Characterization of the ΔaraC. **(A)** Genetic stability detection of deletion mutant HY9901ΔaraC. M: DL2000 marker; Lane 1, a fragment of 711 bp is obtained for HY9901 using primer pairs of araC-F/araC-R. Lane 2, the result was negative, and is obtained for HY9901ΔaraC using primer pairs of araC-F/araC-R. **(B)** Growth rates of HY9901ΔaraC and HY9901. Aliquots of cell culture were taken at various time points and measured for cell density at OD600. **(C)** Measurement of biofilm by crystal violet ammonium oxalate. **(D)** Measurement of biofilm by LSCM. a: HY9901ΔaraC 2.5d diagram; b: HY9901 2.5d diagram; c: HY9901ΔaraC 2d diagram; d: HY9901 2d diagram; HY9901 Biofilm thickness: 120 ± 20 μm; HY9901ΔaraC Biofilm thickness: 160 ± 20 μm.

TABLE 5 | Drug sensitivity test results of the HY9901 and HY9901Δ*araC*.

Antibiotic	Dose (μg)	Bacteriostatic circle diameter (mm)			
		HY9901	Sensitivity[a]	Δ*araC*	Sensitivity
Cefperazone	75	0	R	0	R
Oxacillin	1	0	R	0	R
Clindamycin	2	0	R	0	R
Ceftazidime	30	0	R	0	R
Penicillin	10 U	0	R	0	R
Ampicillin	10	0	R	0	R
Caebenicillin	100	0	R	0	R
Cefazolin	30	0	R	9.5 ± 0.2	R
Ceftriaxone	30	0	R	0	R
Cephradine	30	0	R	0	R
Piperacillin	100	0	R	0	R
Cefuroxime	30	0	R	0	R
SMZ/TMP	23.75/1.25	0	R	0	R
Aboren	30	0	R	11.0 ± 0.2	R
Vancomycin	30	0	R	0	R
Cephalexin	30	0	R	0	R
Polymyxin B	200 IU	0	R	0	R
Norfloxacin	10	0	R	0	R
Ofloxacin	5	0	R	0	R
Ciprofloxacin	5	0	R	0	R
Amikacin	30	11.0 ± 0.2	R	14.5 ± 0.3	I
Minocyline	30	13.5 ± 0.2	R	19.0 ± 0.4	S
Tetracyline	30	12.1 ± 0.1	R	15.9 ± 0.3	I
Gentamicin	10	0	R	16.0 ± 0.2	S
Furazolidone	300	7.1 ± 0.1	R	9.5 ± 0.2	R
Chloramphenicol	30	14.5 ± 0.3	I	21.0 ± 0.4	S
Kanamycin	30	0	R	12.1 ± 0.2	R
Erythromycin	15	0	R	10.5	R
Doxycycline	30	10.5 ± 0.1	S	19.0 ± 0.3	S
Cefperazone	30	0	R	13.6 ± 0.2	I

[a]*S (susceptible), I (intermediate), R (resistant).*

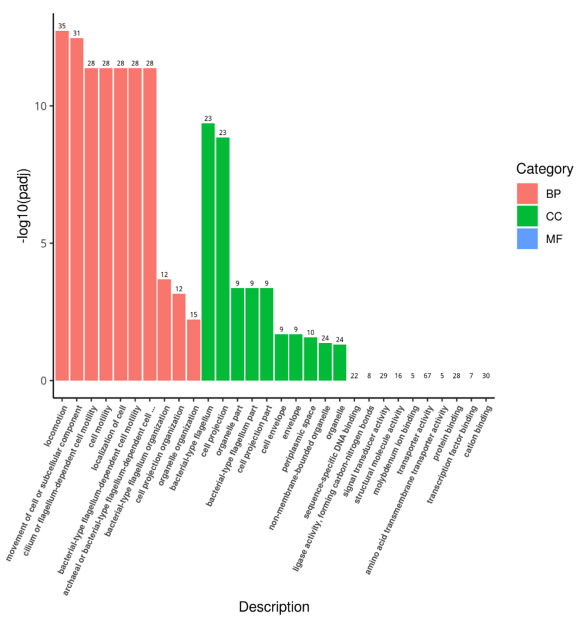

FIGURE 3 | GO enrichment analysis histogram.

the expression of *vopN*, *vopS*, and *vcrV* (6, 12, 24, 48, and 72 h) ($p < 0.01$) (**Figure 4**).

Expression and Secretion of HopPmaJ Is Regulated by AraC

Western Blot Analysis

The mouse anti-serum to recombinant HopPmaJ rabbit antiserum of *V. alginolyticus* HY9901 strain was analyzed by Western blot with the whole bacterial protein extracted from HY9901 and Δ*araC*. The results showed that the protein recognition and the deletion of *araC* gene significantly reduced the secretion of HopPmaJ protein (12.78 kDa) (**Figure 5A**).

β-Galactosidase Assay

In this experiment, the recombinant plasmid pME6522-*hop-lacZ* was introduced into HY9901 and Δ*araC*. The lacZ reporter gene fusion experiment was used to study the regulatory relationship between the regulatory factor AraC and HopPmaJ protein (**Figure 5B**), which can be seen from the results detected in HY9901. β-Galactosidase activity was statistically significantly higher than Δ*araC* and has a significant difference ($p < 0.05$), indicating that AraC can activate the expression of *hop* gene.

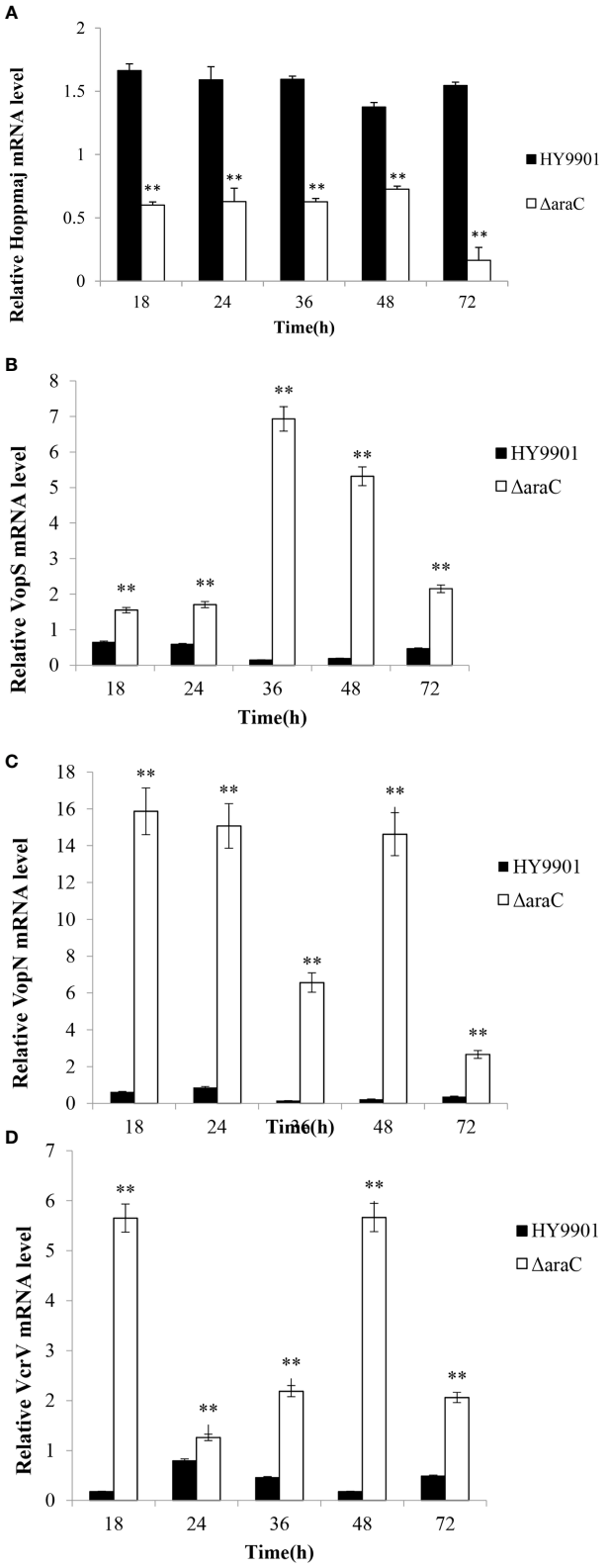

FIGURE 4 | Expression of HY9901 and HY9901Δ*araC* T3SS-related genes induced by DMEM. **(A)** Relative HopPmaJ mRNA level. **(B)** Relative VopS mRNA level. **(C)** Relative VopN mRNA level. **(D)** Relative VcrV mRNA level. *P < 0.05, **P < 0.01.

Safety Assessment

The mutant strain Δ*araC* can survive in zebrafish. After 2 days of infection, Δ*araC* reaches the maximum concentrations in the liver and spleen, then gradually decreases and disappears on the 7th day (**Figure 6**), indicating that the live-attenuated vaccine is safe for zebrafish.

Vaccine Efficacy

Fish vaccinated with Δ*araC* were challenged 4 weeks post vaccination with HY9901. Within 14 days, the mortality rate in the control group injected with sterile PBS was 90%, the mortality rate in the injection immunization group was 35%, and the relative percentage survival was 61.3% (**Figure 7**).

Histological Sections and Pathological Observation of Spleen and Liver of Zebrafish

In the tissue sections, the fish infected with wild strain HY9901 showed symptoms such as hyperemia and blurred lymphocyte boundaries, and a small amount of hyperemia in the liver tissue of the immune group, but not in the negative control group (**Figure 8**).

Immune Gene Expression of Zebrafish Induced by HY9901Δ*araC* Vaccine

Detection of immune gene expression in zebrafish immunized with HY9901Δ*araC* live-attenuated vaccine was assessed by qPCR to analyze the transcriptional levels of pro-inflammatory and immunoglobulin-related immune genes. The results showed that the group vaccinated with HY9901Δ*araC* had a significantly increased expression of *il-6*, *il-6R*, *il-1β*, *il-8*, *igm*, *gata-1*, *lyz*, *rag-1*, *tlr5*, and *c/ebpβ* genes in liver and *il-6*, *il-6R*, *il-1β*, *igm*, *gata-1*, *lyz*, *rag-1*, *tlr5*, and *c/ebpβ* genes in spleen compared to control fish injected with PBS ($p < 0.01$) (**Figure 9**).

DISCUSSION

T3SS is an important virulence mechanism of Gram-negative bacteria, which has attracted much attention because it participates in the interaction between bacteria and host. The secretion of T3SS virulence proteins is directly or indirectly regulated by the AraC/XylS family of transcription factors in an environment-specific manner (Hueck, 1998; Hu et al., 2017). In *Vibrio cholerae*, the overall structure of ToxT is similar to that of AraC protein, and it can directly regulate virulence genes (Li et al., 2016a; Narm et al., 2020), but there is no research on the regulatory function of AraC in *V. alginolyticus*.

In this study, we obtained the mutant strain Δ*araC* of *V. alginolyticus*, explored its biological characteristics and pathogenicity, and evaluated its potential as a live-attenuated vaccine. The extracellular products (ECPs) produced by *V. alginolyticus* have a variety of extracellular proteases that represent important virulence factors (Hare et al., 1983). Lee et al. (1996) reported that they isolated an alkaline serine protease from

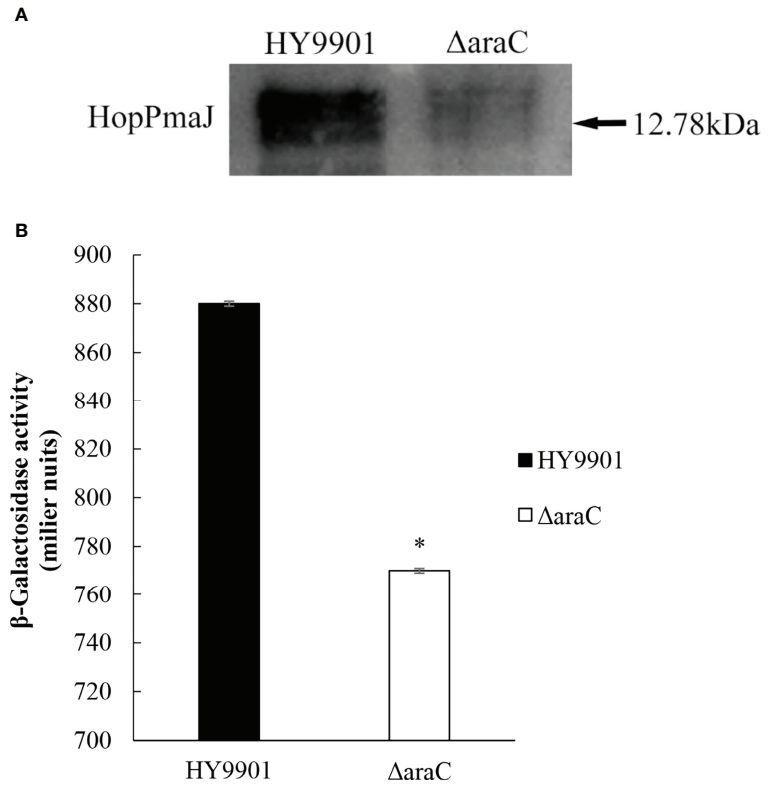

FIGURE 5 | (A) Western blot showing the specificity for the polyclonal antibody against HopPmaJ (12.78 kDa) for the Δ*araC* strain (right lane) and wild strain HY9901 (left lane). **(B)** *β*-Galactosidase activity from the *hop* promoter. Promoter activity of *hop* in HY9901 and Δ*araC*. Bacteria were grown in TSB, and levels of *β*-galactosidase activity were measured (*y*-axis). The values are the average of three separate experiments, and the standard deviation is indicated by error bars. Asterisks represent statistical difference ($p < 0.05$).

the ECP of *V. alginolyticus*, which can decompose and destroy the host tissue, resulting in fish disease (Lee et al., 1996). (Zhou et al., 2013) reported that there was no significant difference in growth rate, extracellular protease activity, and biofilm-forming ability after the deletion of *vscO* gene in *V. alginolyticus* HY9901 (Zhou et al., 2013). The mutant strain Δ*sodB* of *V. alginolyticus* also showed no significant difference in growth rate, but its ability to form biofilm was enhanced (Chen et al., 2019). Biofilms, matrix-encapsulated microbial aggregates, attach to biological or abiotic surfaces, and are resistant to antibiotics (Costerton et al., 1999; Hall-Stoodley et al., 2004). Biofilm formation is the function of self-generated EPS matrix wrapping biofilm

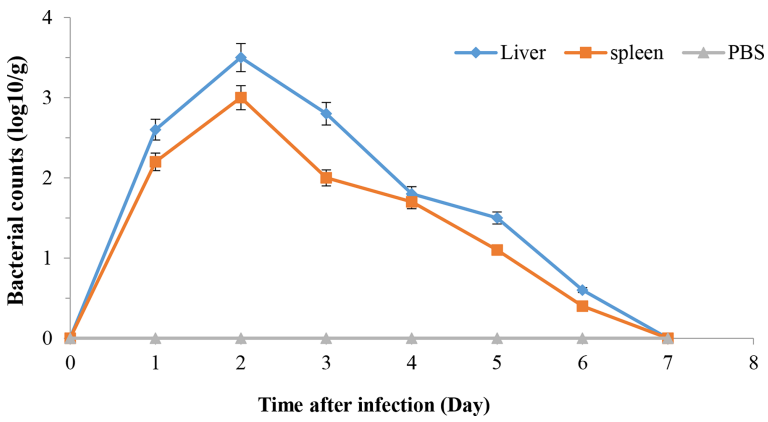

FIGURE 6 | Propagation of HY9901Δ*araC* in zebrafish liver and spleen following i.m. injection with 5 μl of 1 × 10^5 cfu ml^{-1} Δ*araC*. Control fish were i.m. injection with 5 μl of sterile PBS. The number of viable bacteria was shown as the mean ± standard of three samples.

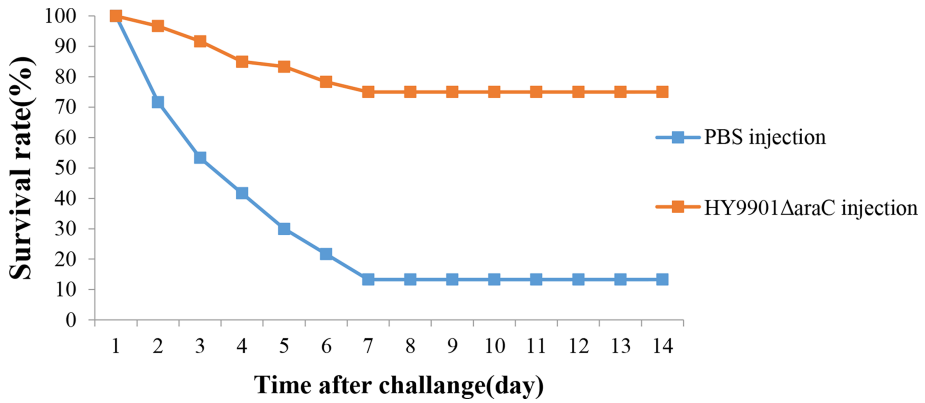

FIGURE 7 | Survival in groups vaccinated with HY9901ΔaraC and PBS following challenge with Vibrio alginolyticus HY9901.

cells, which are mainly composed of polysaccharides, proteins, lipids, and extracellular DNA (Flemming et al., 2016). Similarly, as found in this study, there was no significant difference in the growth rate and extracellular enzyme activity of HY9901Δ*araC* when compared with the wild-type strain HY9901. These results suggested that gene *araC* may not affect these characteristics of *V. alginolyticus*. In addition, only at 24 h was the aggregation ability of biofilm significantly enhanced over that of the wild-type strain HY9901, and it was basically the same at other times. During the pathogenic process of bacteria, density sensing systems can regulate the formation of biofilm (Scutera et al., 2014). Therefore, AraC protein significantly affects the biofilm thickness during the stable period of bacterial growth, which may be related to the phenomenon of density sensing, but this needs further experimental verification.

The most important determinants of *V. alginolyticus* invasion and colonization are biofilms and motility (He et al., 2011). Bacterial motility is achieved *via* a moving organelle, the flagella (Sourjik and Wingreen, 2012). The flagellum is a spiral filament composed of flagellin (Abram and Koffler, 1964). The synthesis and movement of *Vibrio harveyi* flagella are controlled by a quorum sensing system, which inhibits the motility and reduces the toxicity to brine shrimp larvae. This highlights that motility is related to the virulence of *Vibrio* (Echazarreta and Klose, 2019). There are also many studies on the motility and virulence of *V. alginolyticus*. For example, the deletion of T3SS chaperone proteins *vscO* gene will weaken the swimming ability and reduce the virulence to fish by 10 times Zhou et al. (2013). The deletion of the effector protein expressed by *hop* gene also causes an impairment of swimming ability and decreased the virulence

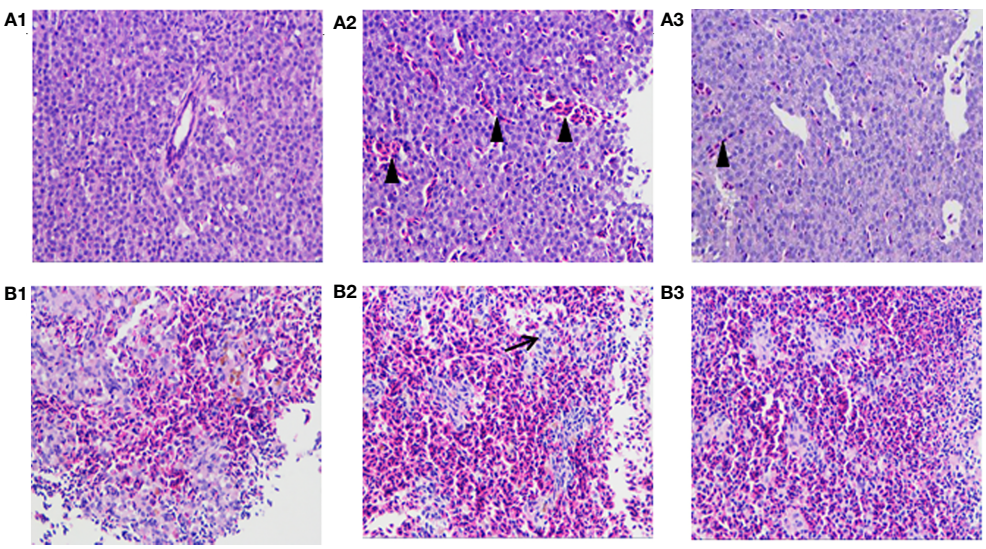

FIGURE 8 | Histological sections of spleen and liver of zebrafish. Zebrafish liver tissue section (A1: PBS liver tissue, A2: HY9901 liver tissue, A3: ΔaraC liver tissue); zebrafish spleen tissue section (B1: PBS spleen tissue, B2: HY9901 spleen tissue, B3:ΔaraC spleen tissue). The triangle (▲) represents hyperemia, and the arrow (↗) represents the blurred boundary of lymphocytes in the figure.

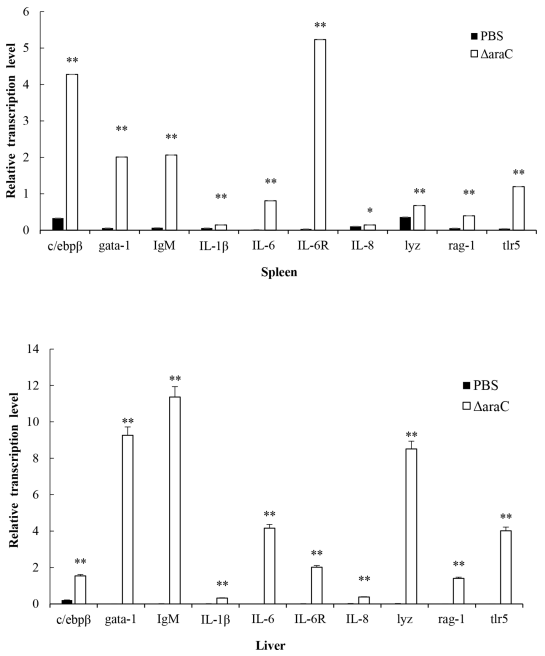

FIGURE 9 | Comparative analysis of the expression of immune-related genes in liver and spleen of zebrafish given the live-attenuated vaccine and unvaccinated zebrafish. The head, kidney, and spleen of grouper were sampled at 1 day before challenge, and the mRNA level of each immune-related gene was normalized to that of *β*-actin. Bars represent the mean relative expression of three biological replicates and error bars represent standard deviation. *P < 0.05, **P < 0.01.

to grouper by 2,600 times (Pang et al., 2018). The deletion of the regulatory protein expressed by *tyeA* gene lessened the swimming ability and attenuated the virulence to zebrafish by nearly 40 times. In addition, qRT-PCR showed that the deletion of apparatus proteins *vscQ* gene downregulated the expression of flagellin genes *flaA* and *fliG*, and the absence of flagellin will affect the normal growth and movement of flagellum (Millikan and Ruby, 2004), which was the reason why it showed a weak population phenotype and reduced its virulence to zebrafish by 4.6 times (Wu et al., 2020). In this study, Δ*araC* significantly reduced its swimming ability and toxicity to zebrafish, and transcriptome data showed that *araC* gene is closely related to movement, flagellum switch, and flagellum composition. The decline of virulence and swimming ability may be related to bacterial flagella. These results suggest that *araC* gene may affect the swimming ability and virulence of *V. alginolyticus* by regulating the expression of flagella-related genes, but its specific regulatory mechanism needs to be further studied.

The regulation of T3SS is mainly achieved through the regulation of the expression and secretion of its effector proteins, which have been demonstrated in many independent studies *in vitro* and *in vivo*. In *P. aeruginosa*, ExsA is the central transcriptional regulator of T3SS (Hovey and Frank, 1995), which controls the expression of secretory apparatus translocation mechanism and secreted effector proteins, and ExsD is the anti-activator of ExsA. McCaw et al. (2002) showed that the deletion of *exsD* gene significantly promoted the secretion of T3SS and the expression of regulatory protein and effector protein through a lacZ reporter gene fusion experiment during which β-galactosidase activity was assayed (McCaw et al., 2002). In *V. parahaemolyticus*, ExsA and ExsD are also the main positive and negative regulators of T3SS1 (Zhou et al., 2010). The expression and secretion of T3SS1 are inseparable from ExsA, and EMSA experiments showed that the ExsA protein can directly interact with the promoter sequence of effector protein Vp1687. In addition, the destruction of the regulatory protein *exsD* gene or the overexpression of *exsA* will also lead to the constructive expression of T3SS1 gene and the secretion of effector protein Vp1656 (Zhou et al., 2008). Liu et al. (2016) found that *V. alginolyticus* ExsA plays a positive regulatory role on effector proteins Va1686 and Va1687 (Liu et al., 2016). Zhou et al. (2020) showed that TyeA is the regulatory protein of *V. alginolyticus* T3SS. In the absence of *tyeA* gene, the mRNA expression of effector proteins VopS, VopN, and HopPmaj was significantly upregulated (Zhou et al., 2020). In this study, the deletion of *araC* gene also increased the mRNA expression of VopS, VcrV, and VopN, but reduced the mRNA expression of HopPmaJ. Western blotting and β-galactosidase reporter gene experiments had also verified that AraC could activate the expression of HopPmaJ. AraC may regulate the transcription of T3SS-related genes in some way, so as to increase and reduce the expression of effector proteins of *V. alginolyticus*. However, the specific regulation mechanism needs to be further evaluated. This study laid a foundation for the follow-up studies of T3SS regulation mechanisms.

The mechanism of bacterial drug resistance is complex; in addition to the antagonistic effects of biofilm on antibiotics (Stewart, 2001), the drug resistance of bacteria can also be obtained through the transmission of drug-resistant plasmid conjugation and transduction (Zhou et al., 2018). Furthermore, gene mutation will spontaneously produce a new resistance characteristic (Levy, 1998) or, from other iron carriers, carry drug resistance genes. In addition, *V. alginolyticus* also carries drug resistance genes. Raissy et al. (2012) tested 20 strains of *V. alginolyticus* (from seafood including fish, shrimp, lobster, and crab from cages off the Persian Gulf) for drug sensitivity and PCR detection. The results showed that several strains of *V. alginolyticus* could possess *strB*, *tetS*, and *ermB* genes, which encode streptomycin, tetracycline, and erythromycin resistance, respectively and some strains did not contain *tetS* gene. However, resistance to tetracycline may be due to the existence of other genes encoding tetracycline resistance, such as *tetA*, *tetB*, *tetM*, and *tetK* (Raissy et al., 2012). In this study, the results of drug sensitivity tests showed that *V. alginolyticus* HY9901 was resistant to most antibiotics, while the deleted strain Δ*araC* became sensitive to amikacin, tetracycline, neomycin, minocycline, and gentamicin. We speculated that the deletion mutation of *araC* gene may lead to new drug resistance characteristics, or AraC protein may regulate the expression of drug resistance genes. The specific drug resistance mechanism needs to be further studied.

Drug resistance carried by bacteria will flow from the food chain into human body *via* aquatic products (Chen et al., 2020). Human infection with drug-resistant bacteria will limit the choice of antibiotics, resulting in difficult treatment, which poses

a threat to human health (Shahimi et al., 2021). Therefore, it is necessary to find alternative methods to control *V. alginolyticus* strains harmful to aquatic animals. Production of a live-attenuated vaccine provides a promising potential prophylactic tool for controlling bacterial fish diseases (Zhang et al., 2013). Vaccines play a major role by reducing or eliminating the risk of infection caused by antibiotic-resistant strains. This can be achieved by antibody-mediated killing of organisms in the blood (Klugman and Black, 2018). In terms of immune protection and production, live-attenuated vaccines have advantages over inactivated vaccines (Chen et al., 2020). Because zebrafish produces fast and many immune-related genes have been found, it is a model suitable for studying immune defense (Randelli et al., 2008). Zhang et al. (2013) found that zebrafish inoculated with live-attenuated *Vibrio anguillarum* vaccine induced good proinflammatory cytokine IL-1β response but the expression chemokine IL-8 gene transcription decreased compared with non-inoculated fish. However, following challenge, the expression level of adaptive immune-related genes increased, indicating that live-attenuated vaccine triggered protection by inhibiting the expression of inflammatory factors post-challenge and enhanced adaptive immunity (Zhang et al., 2013). In this study, the relative survival rate of zebrafish immunized with vaccine candidate Δ*araC* was 61.3%. Evaluation of vaccine safety showed that vaccination of Δ*araC* live-attenuated vaccine did not cause obvious pathological changes or clinical symptoms. Compared with the PBS group, HY9901 Δ*araC* immunization significantly increased the expression of *gata-1*, *il6*, *igm*, *il-1β*, *lyz*, *rag-1*, *tlr5*, and *c/ebpβ* genes in the spleen and liver, possibly relating to protection in zebrafish.

CONCLUSIONS

We analyzed the biological characteristics and pathogenicity of HY9901 mutant strain Δ*araC*. The mutant *V. alginolyticus* strain Δ*araC* did not adversely affect growth and extracellular protease production. Furthermore, the biofilm-forming ability did not change significantly, but weakening of the swimming ability was evident, as well as the subsequent virulence to zebrafish. Wild strains and mutant strains were resistant to most antibiotics. qRT-PCR, Western blotting, and β-galactosidase reporter gene experiments showed that AraC could upregulate the expression of the T3SS effector protein HopPmaJ. As a live-attenuated vaccine, it is protective against *V. alginolyticus* and can induce protective immune responses in zebrafish. These results provide a reference for the study of the regulatory mechanisms of the regulatory protein on T3SS. This study provides further evidence of immune efficacy and safety of live-attenuated bacterial vaccines for potential use in aquaculture. Such data are imperative for evidence-based support for the technology's application in the prevention and control of fish diseases caused by *V. alginolyticus*.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Ethics Committee of Guangdong Ocean University (Zhanjiang, China).

AUTHOR CONTRIBUTIONS

All the authors have made extensive contributions to the work in this manuscript. JW and HF designed the experiment; JW, FY and HF generated experimental data and wrote manuscripts. HF, LC, WZ, and XX assisted in completing the experiment. HP, SM, JJ, and NW conceived the work and critically reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.938742/full#supplementary-material

REFERENCES

Abram, D. and Koffler, H. (1964). *In Vitro* Formation of Flagella-Like Filaments and Other Structures From Flagellin. *J. Mol. Biol.* 9, 168–185. doi: 10.1016/s0022-2836(64)80098-x

Aly, S. M., Eissa, A. E., Elbanna, N. I. and Albutti, A. (2021). Efficiency of Monovalent and Polyvalent *Vibrio Alginolyticus* and *Vibrio Parahaemolyticus* Vaccines on the Immune Response and Protection in Gilthead Sea Bream, *Sparus Aurata* (L.) Against Vibriosis. *Fish Shellfish Immunol.* 111, 145–151. doi: 10.1016/j.fsi.2020.10.011

Bunpa, S., Chaichana, N., Teng, J. L. L., Lee, H. H., Woo, P. C. Y., Sermwittayawong, D., et al. (2020). Outer Membrane Protein A (OmpA) is a Potential Virulence Factor of *Vibrio Alginolyticus* Strains Isolated From Diseased Fish. *J. Fish Diseases*. 43, 275–284. doi: 10.1111/jfd.13120

Bunpa, S., Nishibuchi, M., Thawonsuwan, J. and Sermwittayawong, N. (2018). Genetic Heterogeneity Among *Vibrio Alginolyticus* Strains, and Design of a PCR-Based Identification Method Using *gyrB* Gene Sequence. *Can. J. Microbiol.* 64, 1–10. doi: 10.1139/cjm-2017-0269

Cai, X. H., Peng, Y. H., Wang, Z. C., Huang, T., Xiong, X. Y., Huang, Y. C., et al. (2016). Characterization and Identification of Streptococci From Golden Pompano in China. *Dis. Aquat Organ.* 119, 207–217. doi: 10.3354/dao02998

Cai, S. H., Wu, Z. H., Jian, J. C. and Lu, Y. S. (2007). Cloning and Expression of Gene Encoding the Thermostable Direct Hemolysin From *Vibrio Alginolyticus* Strain HY9901, the Causative Agent of Vibriosis of Crimson Snapper (*Lutjanus Erythopterus*). *J. Appl. Microbiol.* 103, 289–296. doi: 10.1111/j.1365-2672.2006.03250.x

Chen, J. M., Sun, R. X., Pan, C. G., Sun, Y., Mai, B. X. and Li, Q. X. (2020). Antibiotics and Food Safety in Aquaculture. *J. Agric. Food Chem.* 68, 11908–11919. doi: 10.1021/acs.jafc.0c03996

Chen, Y., Wu, F., Pang, H., Tang, J., Cai, S. and Jian, J. (2019). Superoxide Dismutase B (*Sodb*), an Important Virulence Factor of Vibrio Alginolyticus, Contributes to Antioxidative Stress and its Potential Application for Live Attenuated Vaccine. *Fish Shellfish Immunol.* 89, 354–360. doi: 10.1016/j.fsi.2019.03.061

Costerton, J. W., Stewart, P. S. and Greenberg, E. P. (1999). Bacterial Biofilms: A Common Cause of Persistent Infections. *Sci. (New York N.Y.).* 284, 1318–1322. doi: 10.1126/science.284.5418.1318

Ding, C. C., Liu, Q., Li, J., Ma, J. F., Wang, S. Y., Dong, Q. L., et al. (2019). Attenuated Listeria Monocytogenes Protecting Zebrafish (*Danio Rerio*) Against *Vibrio* Species Challenge. *Microbial Pathogenesis.* 132, 38–44. doi: 10.1016/j.micpath.2019.03.040

Echazarreta, M. A. and Klose, K. E. (2019). *Vibrio* Flagellar Synthesis. *Front. Cell. Infection Microbiol.* 9. doi: 10.3389/fcimb.2019.00131

Flemming, H. C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S. A. and Kjelleberg, S. (2016). Biofilms: An Emergent Form of Bacterial Life. *Nat. Rev. Microbiol.* 14, 563–575. doi: 10.1038/nrmicro.2016.94

Guennoc, C.M., Rose, C., Guinnet, F., Miquel, I., Labbe, J. and Deveau, A. (2017). A New Method for Qualitative Multi-Scale Analysis of Bacterial Biofilms on Filamentous Fungal Colonies Using Confocal and Electron Microscopy. *J. Vis. Exp*. 119, 54771. doi: 10.3791/54771

Hall-Stoodley, L., Costerton, J. W. and Stoodley, P. (2004). Bacterial Biofilms: From the Natural Environment to Infectious Diseases. *Nat. Rev. Microbiol.* 2, 95–108. doi: 10.1038/nrmicro821

Hare, P., Scott-Burden, T. and Woods, D. R. (1983). Characterization of Extracellular Alkaline Proteases and Collagenase Induction in *Vibrio Alginolyticus*. *J. Gen. Microbiol.* 129, 1141–1147. doi: 10.1099/00221287-129-4-1141

He, H. H., Wang, Q. Y., Sheng, L. L., Liu, Q. and Zhang, Y. X. (2011). Functional Characterization of *Vibrio Alginolyticus* Twin-Arginine Translocation System: Its Roles in Biofilm Formation, Extracellular Protease Activity, and Virulence Towards Fish. *Curr. Microbiol.* 62, 1193–1199. doi: 10.1007/s00284-010-9844-6

Hovey, A. K. and Frank, D. W. (1995). Analyses of the DNA-Binding and Transcriptional Activation Properties of ExsA, the Transcriptional Activator of the *Pseudomonas Aeruginosa* Exoenzyme S Regulon. *J. bacteriology.* 177, 4427–4436. doi: 10.1128/jb.177.15.4427-4436.1995

Hueck, C. J. (1998). Type III Protein Secretion Systems in Bacterial Pathogens of Animals and Plants. *Microbiol. Mol. Biol. Rev.* 62, 379–433. doi: 10.1128/MMBR.62.2.379-433.1998

Hu, Y. M., Huang, H., Cheng, X., Shu, X. S., White, A. P., Stavrinides, J., et al. (2017). A Global Survey of Bacterial Type III Secretion Systems and Their Effectors. *Environ. Microbiol.* 19, 3879–3895. doi: 10.1111/1462-2920.13755

Kinoshita, M., Shimizu, M., Akiyama, K., Kato, H., Moriyama, K. and Sawa, T. (2020). Epidemiological Survey of Serum Titers From Adults Against Various Gram-Negative Bacterial V-Antigens. *PloS One* 15e0220924. doi: 10.1371/journal.pone.0220924

Klugman, K. P. and Black, S. (2018). Impact of Existing Vaccines in Reducing Antibiotic Resistance: Primary and Secondary Effects. *Proc. Natl. Acad. Sci. U. S. A.* 115, 12896–12901. doi: 10.1073/pnas.1721095115

Lee, K. K., Yu, S. R., Yang, T. I., Liu, P. C. and Chen, F. R. (1996). Isolation and Characterization of *Vibrio Alginolyticus* Isolated From Diseased Kuruma Prawn, *Penaeus Japonicus*. *Lett. Appl. Microbiol.* 22, 111–114. doi: 10.1111/j.1472-765x.1996.tb01121.x

Levy, S. B. (1998). The Challenge of Antibiotic Resistance. *Sci. American.* 278, 46–53. doi: 10.1038/scientificamerican0398-46

Liu, J. X., Lu, S. Y., Orfe, L. H., Ren, C. H., Hu, C. Q., Call, D. R., et al. (2016). ExsE Is a Negative Regulator for T3SS Gene Expression in *Vibrio Alginolyticus*. *Front. Cell. Infection Microbiol.* 6. doi: 10.3389/fcimb.2016.00177

Liu, A. C. and Thomas, N. A. (2015). Transcriptional Profiling of *Vibrio Parahaemolyticus exsA* Reveals a Complex Activation Network for Type III Secretion. *Front. Microbiol.* 6. doi: 10.3389/fmicb.2015.01089

Li, J. Q., Wehmeyer, G., Lovell, S., Battaile, K. P. and Egan, S. M. (2016a). 1.65 Angstrom Resolution Structure of the AraC-Family Transcriptional Activator ToxT From *Vibrio Cholerae*. *Acta Crystallographica Section F-Structural Biol. Commun.* 72, 726–731. doi: 10.1107/s2053230x1601298x

Li, X. C., Xiang, Z. Y., Xu, X. M., Yan, W. H. and Ma, J. M. (2009). Endophthalmitis Caused by *Vibrio Alginolyticus*. *J. Clin. Microbiol.* 47, 3379–3381. doi: 10.1128/jcm.00722-09

Li, W., Yao, Z., Sun, L., Hu, W., Cao, J., Lin, W., et al. (2016b). Proteomics Analysis Reveals a Potential Antibiotic Cocktail Therapy Strategy for Aeromonas Hydrophila Infection in Biofilm. *J. Proteome Res.* 15, 1810–1820. doi: 10.1021/acs.jproteome.5b01127

Lowden, M. J., Skorupski, K., Pellegrini, M., Chiorazzo, M. G., Taylor, R. K. and Kull, F. J. (2010). Structure of *Vibrio Cholerae* ToxT Reveals a Mechanism for Fatty Acid Regulation of Virulence Genes. *Proc. Natl. Acad. Sci. United States America.* 107, 2860–2865. doi: 10.1073/pnas.0915021107

McCaw, M. L., Lykken, G. L., Singh, P. K. and Yahr, T. L. (2002). ExsD is a Negative Regulator of the *Pseudomonas Aeruginosa* Type III Secretion Regulon. *Mol. Microbiol.* 46, 1123–1133. doi: 10.1046/j.1365-2958.2002.03228.x

Millikan, D. S. and Ruby, E. G. (2004). *Vibrio Fischeri* Flagellin A is Essential for Normal Motility and for Symbiotic Competence During Initial Squid Light Organ Colonization. *J. Bacteriology.* 186, 4315–4325. doi: 10.1128/jb.186.13.4315-4325.2004

MO, F., Afriyie, G., Hu, J., Wang, J., Zhou, S., Wang, Z., et al. (2021). Molecular Cloning and Bioinformatics Analysis of *araC* Gene of *Vibrio Alginolyticus*. *Asian Agric. Res.* 13, 19–23. doi: 10.19601/j.cnki.issn1943-9903.2021.02.005

Narm, K. E., Kalafatis, M. and Slauch, J. M. (2020). HilD, HilC, and RtsA Form Homodimers and Heterodimers To Regulate Expression of the *Salmonella* Pathogenicity Island I Type III Secretion System. *J. Bacteriology.* 202, e00012-20. doi: 10.1128/jb.00012-20

O'Toole, G. A. (2011). Microtiter Dish Biofilm Formation Assay. *Jove-Journal Visualized Experiments*. doi: 10.3791/2437

Osorio, C. R. (2018). T3SS Effectors in Vibrios: Homology in Sequence, Diversity in Biological Functions? *Virulence* 9, 721–723. doi: 10.1080/21505594.2018.1435965

Pang, H. Y., Chang, Y. S., Zheng, H. W., Tan, H. M., Zhou, S. H., Zeng, F. Y., et al. (2022). A Live Attenuated Strain of HY9901Δ*vscB* Provides Protection Against *Vibrio Alginolyticus* in Pearl Gentian Grouper (♀ *Epinephelus Fuscoguttatus* × ♂*Epinephelus Lanceolatu*). *Aquaculture* 546737353. doi: 10.1016/j.aquaculture.2021.737353

Pang, H. Y., Qiu, M. S., Zhao, J. M., Hoare, R., Monaghan, S. J., Song, D. W., et al. (2018). Construction of a *Vibrio Alginolyticus* Hoppmaj (*Hop*) Mutant and Evaluation of its Potential as a Live Attenuated Vaccine in Orange-Spotted Grouper (*Epinephelus Coioides*). *Fish Shellfish Immunol.* 76, 93–100. doi: 10.1016/j.fsi.2018.02.012

Pearson, M. M. (2019). Methods for Studying Swarming and Swimming Motility. *Methods Mol. Biol. (Clifton N.J.).* 2021, 15–25. doi: 10.1007/978-1-4939-9601-8_3

Raissy, M., Moumeni, M., Ansari, M. and Rahimi, E. (2012). Antibiotic Resistance Pattern of Some Vibrio Strains Isolated From Seafood. *Iranian J. Fisheries Sci.* 11, 618–626. doi: 10.4103/0972-124X.99267

Rameshkumar, P., Kalidas, C., Tamilmani, G., Sakthivel, M., Nazar, A. K. A., Maharshi, V. A., et al. (2014). Microbiological and Histopathological Investigations of *Vibrio Alginolyticus* Infection in Cobia Rachycentron Canadum (*Linnaeus* 1766) Cultured in Sea Cage. *Indian J. Fisheries.* 61, 124–127.

Randelli, E., Buonocore, F. and Scapigliati, G. (2008). Cell Markers and Determinants in Fish Immunology. *Fish Shellfish Immunol.* 25, 326–340. doi: 10.1016/j.fsi.2008.03.019

Schleif, R. (2000). Regulation of the L-Arabinose Operon of *Escherichia Coli*. *Trends Genet.* 16, 559–565. doi: 10.1016/s0168-9525(00)02153-3

Scutera, S., Zucca, M. and Savoia, D. (2014). Novel Approaches for the Design and Discovery of Quorum-Sensing Inhibitors. *Expert Opin. Drug Discovery.* 9, 353–366. doi: 10.1517/17460441.2014.894974

Shahimi, S., Elias, A., Abd Mutalib, S., Salami, M., Fauzi, F., Zaini, N. A. M., et al. (2021). Antibiotic Resistance and Determination of Resistant Genes Among Cockle (*Anadara Granosa*) Isolates of *Vibrio Alginolyticus*. *Environ. Sci. pollut. Res.* 28, 44002–44013. doi: 10.1007/s11356-021-13665-4

Sheppard, D. E. and Englesberg, E. (1967). Further Evidence for Positive Control of the L-Arabinose System by Gene *araC*. *J. Mol. Biol.* 25, 443–454. doi: 10.1016/0022-2836(67)90197-0

Shi, J., Zhao, W., Xie, J., Zhu, Y., Pan, Y., Ou, J., et al. (2021). Comparison on the Growth Heterogeneity of *Vibrio Parahaemolyticus* Coupled With Strain Source and Genotype Analyses in Different Oligotrophic Conditions. *J. Food protection.* 84, 1904–1910. doi: 10.4315/jfp-21-089

Simon, R., Priefer, U. and Pühler, A. (1983). A Broad Host Range Mobilization System for *In Vivo* Genetic Engineering: Transposon Mutagenesis in Gram Negative Bacteria. *Bio/Technology* 1, 784–791. doi: 10.1038/nbt1183-784

Singer, J. and Englesberg, E. (1971). Arabinose Transport in *araC*-Strains of *EscherichiA* B-R. *Biochim. Biophys. Acta* 249, 498–505. doi: 10.1016/0005-2736(71)90125-8

Sourjik, V. and Wingreen, N. S. (2012). Responding to Chemical Gradients: Bacterial Chemotaxis. *Curr. Opin. Cell Biol.* 24, 262–268. doi: 10.1016/j. ceb.2011.11.008

Stewart, P. S. (2001). Multicellular Resistance: Biofilms. *Trends Microbiol.* 9, 204. doi: 10.1016/s0966-842x(01)01983-7

Tam, V. C., Suzuki, M., Coughlin, M., Saslowsky, D., Biswas, K., Lencer, W. I., et al. (2010). Functional Analysis of VopF Activity Required for Colonization in *Vibrio Cholerae*. *Mbio* 1e00289-10. doi: 10.1128/mBio.00289-10

Wu, P. W., Zhou, S. H., Su, Z. H., Liu, C., Zeng, F. Y., Pang, H. Y., et al. (2020). Functional Characterization of T3SS C-Ring Component VscQ and Evaluation of its Mutant as a Live Attenuated Vaccine in Zebrafish (*Danio Rerio*) Model. *Fish Shellfish Immunol.* 104, 123–132. doi: 10.1016/j.fsi.2020.05.063

Yahr, T. L. and Wolfgang, M. C. (2006). Transcriptional Regulation of the *Pseudomonas Aeruginosa* Type III Secretion System. *Mol. Microbiol.* 62, 631–640. doi: 10.1111/j.1365-2958.2006.05412.x

Yang, J., Hocking, D. M., Cheng, C., Dogovski, C., Perugini, M. A., Holien, J. K., et al. (2013). Disarming Bacterial Virulence Through Chemical Inhibition of the DNA Binding Domain of an AraC-Like Transcriptional Activator Protein. *J. Biol. Chem.* 288, 31115–31126. doi: 10.1074/jbc.M113.503912

Yang, J., Tauschek, M. and Robins-Browne, R. M. (2011). Control of Bacterial Virulence by AraC-Like Regulators That Respond to Chemical Signals. *Trends Microbiol.* 19, 128–135. doi: 10.1016/j.tim.2010.12.001

Yang, B., Zhai, S. Y., Li, X., Tian, J., Li, Q., Shan, H. W., et al. (2021). Identification of *Vibrio Alginolyticus* as a Causative Pathogen Associated With Mass Summer Mortality of the Pacific Oyster (*Crassostrea Gigas*) in China. *Aquaculture* 535, 736363. doi: 10.1016/j.aquaculture.2021.736363

Zhang, Y. L., Tan, H. M., Yang, S. P., Huang, Y. C., Cai, S. H., Jian, J. C., et al. (2022). The Role of *dctP* Gene in Regulating Colonization, Adhesion and Pathogenicity of *Vibrio Alginolyticus* Strain HY9901. *J. Fish Diseases.* 45, 421–434. doi: 10.1111/jfd.13571

Zhang, Z. H., Wu, H. Z., Xiao, J. F., Wang, Q. Y., Liu, Q. and Zhang, Y. X. (2013). Immune Responses Evoked by Infection With *Vibrio Anguillarum* in Zebrafish Bath-Vaccinated With a Live Attenuated Strain. *Veterinary Immunol. Immunopathology.* 154, 138–144. doi: 10.1016/j.vetimm.2013.05.012

Zhou, X. H., Konkel, M. E. and Call, D. R. (2010). Vp1659 Is a *Vibrio Parahaemolyticus* Type III Secretion System 1 Protein That Contributes to Translocation of Effector Proteins Needed To Induce Cytolysis, Autophagy, and Disruption of Actin Structure in HeLa Cells. *J. Bacteriology.* 192, 3491–3502. doi: 10.1128/jb.01493-09

Zhou, Z. J., Pang, H. Y., Ding, Y., Cai, J., Huang, Y. C., Jian, J. C., et al. (2013). VscO, a Putative T3SS Chaperone Escort of *Vibrio Alginolyticus*, Contributes to Virulence in Fish and Is a Target for Vaccine Development. *Fish Shellfish Immunol.* 35, 1523–1531. doi: 10.1016/j.fsi.2013.08.017

Zhou, X. H., Shah, D. H., Konkel, M. E. and Call, D. R. (2008). Type III Secretion System 1 Genes in *Vibrio Parahaemolyticus* are Positively Regulated by ExsA and Negatively Regulated by ExsD. *Mol. Microbiol.* 69, 747–764. doi: 10.1111/j.1365-2958.2008.06326.x

Zhou, S. H., Tu, X. T., Pang, H. Y., Hoare, R., Monaghan, S. J., Luo, J. J., et al. (2020). A T3SS Regulator Mutant of *Vibrio Alginolyticus* Affects Antibiotic Susceptibilities and Provides Significant Protection to *Danio Rerio* as a Live Attenuated Vaccine. *Front. Cell. Infection Microbiol.* 10. doi: 10.3389/fcimb.2020.00183

Zhou, W., Zhu, L. W., Jia, M., Wang, T. C., Liang, B., Ji, X., et al. (2018). Detection of Multi-Drug-Resistant *Escherichia Coli* in a Giant Panda (*Ailuropoda Melanoleuca*) With Extraintestinal Polyinfection. *J. Wildlife Diseases.* 54, 626–630. doi: 10.7589/2017-08-196

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