



A T3SS Regulator Mutant of *Vibrio alginolyticus* Affects Antibiotic Susceptibilities and Provides Significant Protection to *Danio rerio* as a Live Attenuated Vaccine

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Vibrio alginolyticus is a major cause of vibriosis in farmed marine aquatic animals and has caused large economic losses to the Asian aquaculture industry in recent years. Therefore, it is necessary to control *V. alginolyticus* effectively. The virulence mechanism of *V. alginolyticus*, the Type III secretion system (T3SS), is closely related to its pathogenicity. In this study, the T3SS gene *tyeA* was cloned from *V. alginolyticus* wild-type strain HY9901 and the results showed that the deduced amino acid sequence of *V. alginolyticus* *tyeA* shared 75–83% homology with other *Vibrio* spp. The mutant strain HY9901 Δ *tyeA* was constructed by Overlap-PCR and homologous recombination techniques. The HY9901 Δ *tyeA* mutant exhibited an attenuated swarming phenotype and an ~40-fold reduction in virulence to zebrafish. However, the HY9901 Δ *tyeA* mutant showed no difference in growth, biofilm formation and ECPase activity. Antibiotic susceptibility test was observed that wild and mutant strains were extremely susceptible to Amikacin, Minocycline, Gentamicin, Cefperazone; and resistant to oxacillin, clindamycin, ceftazidime. In contrast wild strains are sensitive to tetracycline, chloramphenicol, kanamycin, doxycycline, while mutant strains are resistant to them. qRT-PCR was employed to analyze the transcription levels of T3SS-related genes, the results showed that compared with HY9901 wild type, Δ *tyeA* had increased expression of *vscL*, *vscK*, *vscO*, *vopS*, *vopN*, *vscN*, and *hop*. Following vaccination with the mutant strain, zebrafish had significantly higher survival than controls following infection with the wild-type HY9901 (71.2% relative percent survival; RPS). Analysis of immune gene expression by qPCR showed that vaccination with HY9901 Δ *tyeA* increased the expression of IgM, IL-1 β , IL-6, and TNF- α in zebrafish. This study provides evidence of protective efficacy of a live attenuated vaccine targeting the T3SS of *V. alginolyticus* which may be facilitated by up-regulated pro-inflammatory and immunoglobulin-related genes.

Keywords: *Vibrio alginolyticus*, live attenuated vaccine, type III secretory system, *tyeA*, regulator

INTRODUCTION

Vibrio alginolyticus, a Gram-negative motile rod bacterium, is widely found in seawater and estuaries in various regions of the world (Thompson et al., 2004; Jones et al., 2013). *V. alginolyticus* is highly pathogenic in marine animals (Ben Kahla-Nakbi et al., 2009; Jun et al., 2015) and is associated with diseases of fish, shellfish, shrimp, and coral (Chang et al., 2008; Sadok et al., 2013). It is also a potential zoonotic pathogenic bacteria and incurs great economic losses to the aquaculture industry with raised pressures for the industry to develop controls in recent years. Zoonotic infections can result from human food poisoning causing diarrhea and other symptoms (Li et al., 2009; Sganga et al., 2009). Therefore, it is of great importance to understand the pathogenic mechanisms of *V. alginolyticus* in order to develop an effective vaccine.

Type III secretory system (T3SS) is a highly conserved secretory system in several Gram-negative bacteria such as *Yersinia* spp., *Salmonella* spp., *Vibrio cholerae*, *Pseudomonas* spp. (Anand et al., 2003; Masato et al., 2014; Khavong and Lorena, 2016; Dorothea et al., 2017). When *V. alginolyticus* infects the host, the T3SS can secrete virulence proteins outside the cell or on the surface of the host cell, or even directly inject virulent proteins into the host cell, resulting in cell death (Pallen et al., 2005). Although the T3SS mechanism is usually conserved in Gram-negative pathogens, the functions of its regulator vary widely. Comparative genome analysis showed that the T3SS of *V. alginolyticus* was similar to that of *Vibrio parahaemolyticus* (Martinez-Argudo and Blocker, 2010), but little is known about the regulator TyeA of *V. alginolyticus* T3SS.

Until now, there was no related research on *tyeA* gene in *Vibrio alginolyticus*, and its function was still being explored. It has been found that TyeA is a regulator of T3SS and involved in regulation of effector proteins expression (Sundberg and Forsberg, 2003). Numerous studies have shown that the YopN-TyeA heterodimer plays a particularly important role in regulating the secretion of effector proteins in *Yersinia* (Iriarte et al., 1998; Cheng and Schneewind, 2000; Schubot et al., 2004; Ferracci et al., 2005). In order to understand the function of TyeA in T3SS, we first constructed a mutant of *tyeA* gene, studied the biology and pathogenicity of the HY9901 Δ *tyeA* strain and the transcription levels of T3SS-related genes were then analyzed by qRT-PCR. In addition, we found that HY9901 Δ *tyeA* mutant can be used as an effective live vaccine against *Vibrio alginolyticus* in zebrafish and induces the expression of zebrafish pro-inflammatory and immunoglobulin-related genes.

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. *E. coli* DH5 α , *E. coli* MC1061 (λ pir) (Rubirés et al., 1997), S17-1 (λ Pir) (Simon et al., 2019), and suicide plasmid pRE112 (Edwards et al., 1998)

were preserved in our laboratory. Healthy zebrafish (*Danio rerio*) were purchased from Zhanjiang Aquatic Market, 3–4 cm long and ~0.2 g in weight. The zebrafish were tested by bacteriological recovery tests and kept in seawater in a circulation system at 28°C for 2 weeks before the experiment.

Reagents and Primers

TIANamp Bacteria DNA Kit (Beijing, Tiangen Biotech Co., Ltd.); Easy Pure™ Quick Gel Extraction Kit and Easy Pure™ Plasmid MiniPrep Kit (Beijing, TransGen Biotech Co., Ltd.); pMD18-T vector, ExTaq DNA polymerase, Prime START MHS DNA polymerase, KpnI, SacI, and T4DNA ligase were all purchased from TaKaRa (Japan). The primers were synthesized by Sangon Biotech Co., Ltd.

Cloning and Sequencing of the *tyeA* Gene From *V. alginolyticus* HY9901

A pair of primers *tyeA*P1-F/*tyeA*P1-R were designed as shown in Table 2 according to the *V. alginolyticus* gene sequence (GenBank Number: GU074526.1). PCR was performed in a Thermocycler (Bio-Rad, CA, USA) under the following optimized amplification conditions: an initial denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s. Five microlitre of each amplicon was examined on a 1% agarose gel stained with ethidium bromide. The PCR product was recovered from the agarose gel to ligate into the pMD18-T vector and transformed into *E. coli* DH5 α (Table 1). The inserted fragment was sequenced by Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). Similarity analyses of the determined nucleotide sequences and deduced amino acid sequences were performed by BLAST programs (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and aligned using the program Clustal-X (version 1.81). Protein analysis was conducted with ExPASy tools (<http://expasy.org/tools/>). Location of the domain

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

Strains, plasmids	Relevant characteristics	Source
<i>V. alginolyticus</i> HY9901	Wild type, isolated from diseased <i>Lutjanus sanguineus</i> off the Southern China coast	(Cai et al., 2007)
<i>E. coli</i> DH5 α	supE44 Δ lacU169 (ϕ 80 lacZDM15) hsdR17 recA1 gyrA96 thi-1 relA1	TakaRa
MC1061 (λ pir)	lacY1 galK2 ara-14 xyl-5 supE44 λ pir	(Rubirés et al., 1997)
pRE112	pGP704 suicide plasmid, pir dependent, oriT, oriV, sacB, C mr	(Edwards et al., 1998)
S17-1 (λ pir)	T prSmrrecA thi pro hsdR-M+RP4:2-Tc: Mu: K m T n7 λ pir	(Simon et al., 2019)
S17-1-pRE- Δ <i>tyeA</i>	S17-1 containing plasmid of pRE- Δ <i>tyeA</i> , C mr	This study
pMD18-T	Cloning vector, Amp ^r	TakaRa
Zebrafish	experimental fish, purchased from Zhanjiang Aquatic Market	Zhanjiang Aquatic Market

TABLE 2 | Sequences of primers used in this study.

Primer name	Primer sequence(5'-3')	Accession number
tyeA-for	GGAATCTAGACCTTGAGTCG ATATCTCGACCATCGCGCAA	MN328351
tyeA-int-rev	ATCTTCCCACGCTTCTCTT CAACTTGATAAGCCATAATTCGTCC	
tyeA-int-for	GGACGAATTATGGCTTATCA AGTTGAAGAGGAAGCGTGGGAAGAT	
tyeA-rev	ACAGCTAGCGACGATATGTCA GGCCGGAGGTCATAGAGCT	
tyeA-up	CACATGAACCTCGTTTCGGACTATT	
tyeA-down	TTTCTGGACGCAACAACCTCTGA	
tyeAP1-F	ATGGCTTATCAAGTTTCTA	
tyeAP1-R	TCAATCCAACCTCATCTTCC	
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	TGGACTTCGCAGCACAAATG	AY340959.1
IL-1 β -R	GTTCACTTCACGCTCTTGATG	
IL-8-F	GTCGCTGCATTGAAACAGAA	XM_001342570.2
IL-8-R	CTTAACCCATGGAGCAGAGG	
IgM-F	GTTCTGACCCAGTGACAGAGA	AF246193
IgM-R	CCTGATCACCTCCAGCATAA	
TNF- α -F	TAGAACAACCCAGCAAAC	NC_007130.7
TNF- α -R	ACCAGCGGTAAAGGCAAC	
rag-1-F	GAAGTATACCAAGAAGCCTAAT	NC_007136.7
rag-1-R	TTCCATTATCCTCATCACA	
TLR5-F	GAAACATTACCTGGCACA	NC_007131.7
TLR5-R	CTACAACCAGCACCAACGAATG	
c/ebp β -F	GCCGTACCCAGACTGCTCCGA	NC_007119.7
c/ebp β -R	AGCCGCTTCTTGCCCTTCCC	
β -actin-F	ATGGATGAGGAAATCGCTGCC	NM_131031.1
β -actin-R	CTCCCTGATGTCTGGGTGCTC	
vscL-F	TACCACGGTGAGTGATGTTT	ACY41051.1
vscL-R	CGTAACCGACTTCAGGGA	
hop-F	CTTCGCTTTGCGTTTGCT	KX245315
hop-R	AATACCATCCACCCCTGT	
vscO-F	GAGCTGGAACATTAAGACA	ACY41065.1
vscO-R	TTGCTGCAACTGAACGAA	
vscK-F	GGCGTTATCTCCGTTTCC	ACY41050.1
vscK-R	CTCCGCCCACCATCAATA	
vopN-F	TGAACCTCGTTTCGGACTA	ACY41067.1
vopN-R	ACTTTCTGGAATCGCACT	
vscN-F	TAGGCGAAGAAGGAATGG	ACY41066.1
vscN-R	GCGATAGAAGTGCGCAACAA	
vopS-F	AGTTTTGGAAGTGTTAGCG	ACY41053.1
vopS-R	ACATTGCCTCTGTCATCG	
16S-F	TTGCGAGAGTGAGCGAATCC	NR_044825.2
16S-R	ATGGTGTGACGGGCGGTGTG	

was predicted using the InterProScan program (<http://www.ebi.ac.uk/Tools/pfa/iprscan/>).

Construction of In-frame Deletion Mutant of *tyeA* Gene

According to the method previously described Zujie et al. (2019), Overlap extension PCR was applied to generate an in-frame deletion of the *tyeA* gene on the *V. alginolyticus* wild-type HY9901 chromosome. The in-frame deletion of *tyeA* in the *V. alginolyticus* was generated according to the method of Rubirés et al. (1997). For the construction of Δ *tyeA*, two PCR fragments were generated from HY9901 genomic DNA. The first fragment was amplified using primers tyeA-for (contains a KpnI site at the 5'-end) and tyeA-int-rev, whereas primers tyeA-int-for and tyeA-rev (contains a SmaI site at the 5'-end) were used to amplify the second fragment. Both fragments contained a 20 bp overlapping sequence and were used as templates for the subsequent PCR procedure, which used primers tyeA-for and tyeA-rev. The PCR product was ligated into suicide vector pRE112 (Cmr) to generate pRE- Δ *tyeA*. This recombinant suicide plasmid was transformed into *E. coli* MC1061 λ pir and subsequently S17-1 λ pir. The single crossover mutants were obtained by conjugal transfer of the resulting plasmid into *V. alginolyticus* HY9901. Deletion mutants were screened on 10% sucrose TSA plates. Its presence was subsequently confirmed by PCR and sequencing using primers tyeA-up and tyeA-down.

Characterization of the Δ *tyeA* Genetic Stability of Mutants HY9901 Δ *tyeA*

HY9901 Δ *tyeA* was inoculated onto a TSA plate and passaged blindly for 30 generations. In brief, a single colony was picked from Tryptic Soya agar (TSA) plates and cultured in Tryptic soya broth (TSB, HKM, Guangzhou, China) medium, shaking for 12 h, bacterial broth culture was streaked out on a TSA plate, and this process repeated 30 times. Its genetic stability was determined by the PCR method.

Growth Curve of Bacteria

The wild-type HY9901 strain and the Δ *tyeA* were cultured in TSB for 24 h. The HY9901 and HY9901 Δ *tyeA* were inoculated into TSB at the ratio of 1: 100 (OD₆₀₀ = 0.5) at 28°C, with determination of OD₆₀₀ every 2 h. This procedure was repeated three times per group.

Detection of Extracellular Protease Activity

According to the previously published method (Windle and Kelleher, 1997) wild-type strain HY9901 and HY9901 Δ *tyeA* were coated on TSA plates coated with sterile cellophane, and cultured at 28°C for 24 h, washed with sterile PBS, centrifuged at 4°C for 30 min, and the supernatant filtered to obtain extracellular products. Inactivated sample (supernatant was boiled for 10 min) was used as a blank control.

Swarming Motility

Single colonies of wild-type strain HY9901 and HY9901 Δ *tyeA* were selected by sterile toothpick and plated on TSA plates with agar content of 0.5%. Each group was incubated for 24 h at 28°C. The diameter of swarming circle was measured by Vernier calipers.

Detection of Biofilm Formation Ability Using Crystal Violet Ammonium Oxalate

With reference to the method described previously (Katharine and Watnick, 2003), wild strains HY9901 and HY9901 $\Delta tyeA$ ($OD_{600} = 0.5$) were transferred to a 96-well plate. Each well was inoculated with 200 μ L of bacterium with 6 replicates per sample, the negative control was an inoculum of TSB only, and the culture temperature was 28°C. Samples were taken from the 96 well plate at 12, 24, 48, and 72 h, methanol fixed for 20 min, stained with Crystal violet ammonium oxalate dye for 15 min, rinsed with water and dried. Finally, 95% alcohol was added and incubated at room temperature for 30 min. OD_{570} was determined by Multimode Plate Reader (PerkinElmer EnSpire, EnSpire, Singapore) ($OD_{600} = 1$, bacterial concentration = 1×10^9 cfu/mL).

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

The wild strain HY9901 and the mutant strain HY9901 $\Delta tyeA$ ($OD_{600} = 0.5$) were diluted 50-fold, added to a 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, gently washed three times with physiological saline, and then combined with 10% SYTO9 green. Bacteria were incubated with fluorescent dyes 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 μ m apart, and biofilm parameters—biomass and maximum thickness were determined. Three samples were made for each strain and the average amount calculated.

Dose Response Challenge Test (LD_{50})

The injection concentrations used for the dose response of wild-type strain HY9901 and $\Delta tyeA$ were 10^4 , 10^5 , 10^6 , 10^7 , and 10^8 CFU/mL. A total of 330 fish were randomly divided into three groups (Table 3). The water temperature was adjusted to 28°C. The experiment was repeated three times. Five microlitre of bacterial solution was injected into fish by intramuscular injection. The control group was injected with equivalent volumes of PBS. Fish were monitored for 14 days or until no morbidities occurred. Specific mortality was determined by streaking head-kidney onto TSA. The LD_{50} of the mutant and wild strains was calculated by the Koch method (Reed and Muench, 1938).

Antibiotic Susceptibility

The susceptibility patterns of the HY9901 and HY9901 $\Delta tyeA$ strains to 30 different antibiotics were determined according to the disc diffusion method using TSA (Bauer et al., 1966), and the diameters of the inhibition zones were measured using Vernier calipers. Resistant, intermediate and susceptible phenotype determinations were based on previously published susceptibility paper guidelines (HANGWEI, S1100, China). The strains were inoculated onto TSA plates and allowed to absorb

TABLE 3 | Experiment of LD_{50} .

Concentration HY9901 (CFU/mL)	Death rate (%)	$\Delta tyeA$	Death rate (%)	Control (PBS)	Death rate (%)
10^8	10×3	90	10×3	66.7	—
10^7	10×3	80	10×3	40	—
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

Each experiment involved 110 zebrafish with 10 fish per group in three replicate tanks and the experiment was repeated three times (total fish = 330).

onto agar for 10 min, and antibiotic discs were added after 24 h of incubation at 28°C (Cai et al., 2016).

Expression Analysis of T3SS-Related Genes

T3SS secretion was induced by Dulbecco's Modified Eagle Medium (DMEM) media, and strains HY9901 and HY9901 $\Delta tyeA$ were cultured for 12 h. The primers for T3SS related genes are shown in Table 2 (All primers were designed within the current study). The genes in this study were hop (Pang et al., 2018), vopN, vscN (Yonghong et al., 2016), vscO (Zhou et al., 2013), vopS, vscL, and vscK (Nguyen et al., 2000). 16S rRNA is used as an internal reference. According to the experimental method of Li et al. (2016), RNA was extracted, synthetic cDNA and real-time PCR were used to analyze the expression of T3SS related genes.

Preparation of Vaccine and Vaccination

The $\Delta tyeA$ immune concentration of 10^5 CFU/mL was selected by LD_{50} experiments, which has no mortality to zebrafish (Table 3). The mutant $\Delta tyeA$ was cultured in a shaking flask at 28°C for 18 h, washed and suspended in sterile artificial seawater, and the concentration of the bacterial solution was adjusted to 10^5 CFU mL^{-1} by spectrophotometer. The experimental fish were randomly divided into two groups with 80 fish in each group. The water temperature was adjusted to 28°C. Each fish was injected with 5 μ L $\Delta tyeA$ bacterial solution (10^5 CFU mL^{-1}) by intramuscular injection and control fish were injected with 5 μ L PBS per fish.

Determination of Vaccine Efficacy

Fish were challenged 28 days post vaccination. Thirty fish were randomly selected from each group (in triplicate) and given intramuscular injection of 5 μ L *V. alginolyticus* the wild-type HY9901 (1×10^8 CFU mL^{-1}). The water temperature was adjusted to 28°C and the number of moribund/mortalities was recorded for 14 days. Calculation of the relative percentage survival was performed at the end of the experiment ($RPS (\%) = (1 - \text{immunized group mortality} / \text{control group mortality}) \times 100\%$).

Immune Gene Expression of Zebrafish Induced by HY9901 Δ tyeA Vaccine

Liver and spleen samples were taken from three fish from each group, respectively, 1 day before challenge. Immune-related genes expression levels were detected with real-time qPCR. Primers for IL6, IL6R, IL-1 β , IL-8, IgM, TNF- α , rag-1, TLR5, and c/ebp β are shown in Table 2, and β -actin was used as internal reference. The procedures of RNA extraction, cDNA synthesis, real-time qPCR for analysis of immune gene expression were described by Li et al. (2016).

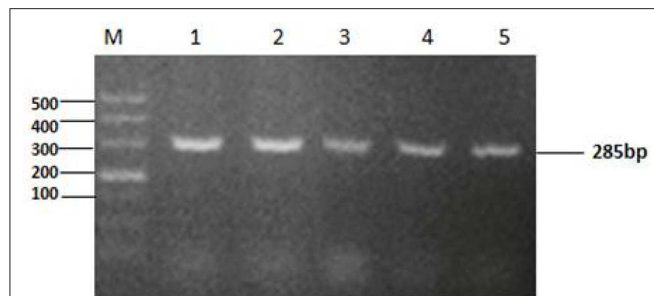


FIGURE 1 | Cloning of *tyeA* gene. M: DL2000 marker. Lane 1-5: The 285bp fragment was amplified from genomic DNA of the wild-type strain HY9901 using primer pairs of *tyeAP1*-for/*tyeAP1*-rev (Lane 1, 2, 3, 4, 5: Tm = 56°C, 57°C, 58°C, 59°C, 60°C).

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 protocols 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 SPSS17.0 software. **indicates extremely significant difference compared with the control group ($p < 0.01$). *indicates significant difference compared with the control group ($p < 0.05$).

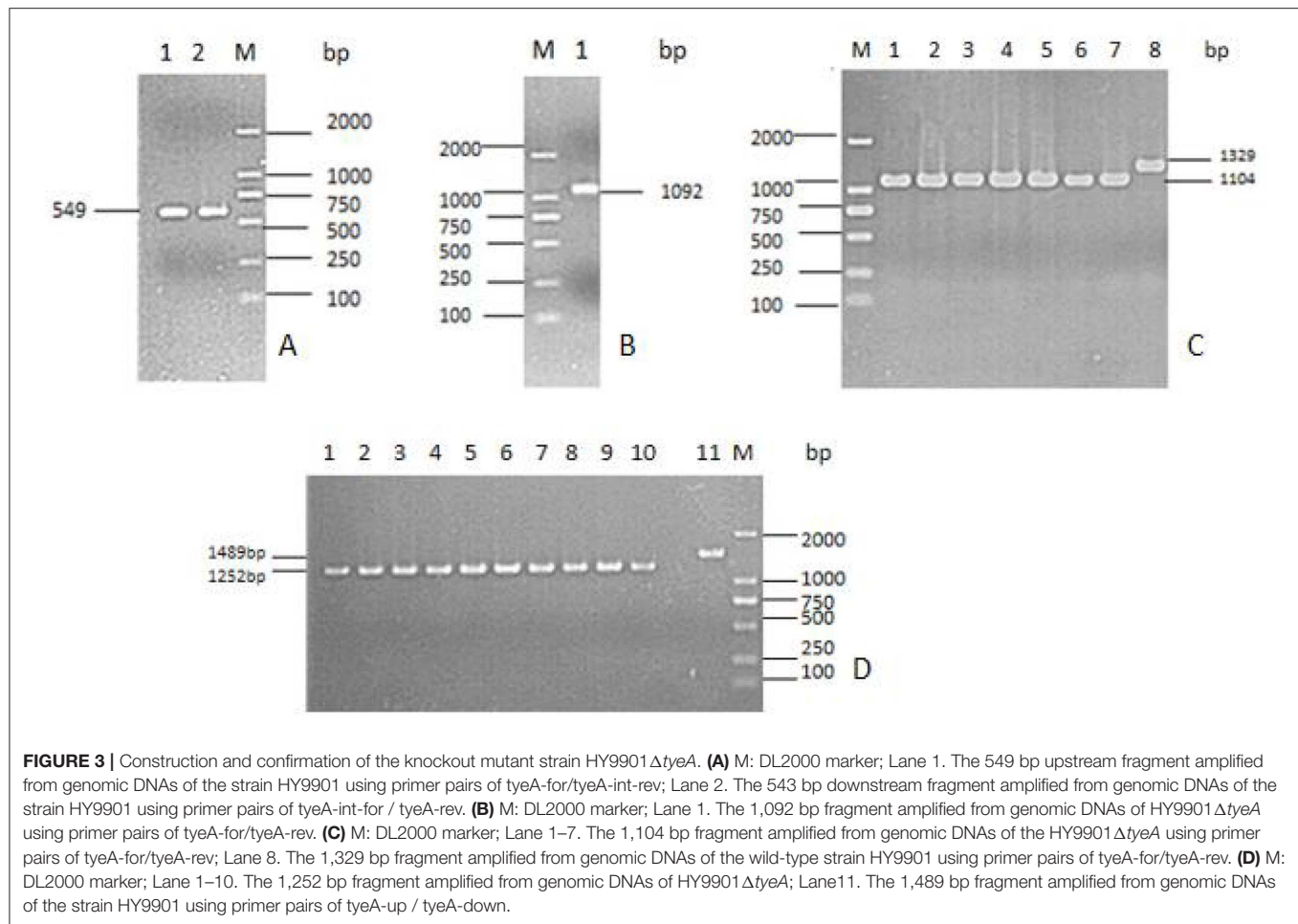
RESULTS

Cloning of *tyeA* Gene and Construction of Mutant

The *tyeA* gene consists of an open reading frame of 285 bp (Figure 1), encoding 95 amino acids (aa) with a predicted molecular weight of 10.98 kDa (*tyeA* accession no. MN328351). The deduced amino acid sequence analysis of TyeA show it has high homology of 75–83% with other *Vibrio* spp. (Figure 2),

<i>Vibrio alginolyticus</i>	MAYQVSNLMADVIALVEQRWVSSDEIWKVANAMELTAVEQ	40		
<i>Vibrio parahaemolyticus</i>	MAYQASDLMDADVIALVEQRWVSSEEIWKIATSMELVAIEQ	40		
<i>Vibrio harveyi</i>	MAYQVTDLMSDVIALVEQRWVGSAEIWNLVNAMEIASMER	40		
<i>Vibrio campbellii</i>	MAYQVTDLMSDVIALVEQRWVGSAEIWNLVNAMEIASMER	40		
<i>Vibrio tubiashii</i>	MAYQDSDLMDIIALVEQRWVGAEAVWKLAESMGLILVEQ	40		
<i>Vibrio bivalvicida</i>	MAYQDSDLMDIIALVEQRWVGAEAVWKLAESMSINSIEQ	40		
<i>Vibrio alginolyticus</i>	KIDFFREFFHKLVRRAIPIIDVFADDEEQRQNLIQAVQKALDEA	80		
<i>Vibrio parahaemolyticus</i>	KIDFFRELHKLIRHIPVDVFADDEEQRQNLIQAAQKALDEA	80		
<i>Vibrio harveyi</i>	KISFFRELHKLIRHIPIDVFNDDEEQRQNLIQAVQKALDEA	80		
<i>Vibrio campbellii</i>	KISFFRELHKLIRHIPIDVFNDDEEQRQNLIQAVQKALDEA	80		
<i>Vibrio tubiashii</i>	KIGFFRELHKLVRHIPVDVFADDEEQRQNLIQAVQKALDEA	80		
<i>Vibrio bivalvicida</i>	KISFFRELHKLVRHIPVDVFADDEEQRQNLIQAVQKALDEA	80		
<i>Vibrio alginolyticus</i>	IDIEEEEAWEDELD	94	Identity	Accession No.
<i>Vibrio parahaemolyticus</i>	IDLEEEEAWEDELD	94	100%	ACY41068.1
<i>Vibrio harveyi</i>	IDLEEEEMWDELD	94	82.98%	ANB96904.1
<i>Vibrio campbellii</i>	IDLEEEEMWDELD	94	78.72%	SQA36766.1
<i>Vibrio tubiashii</i>	VDKEEEEAWEDELD	94	78.72%	AQM67418.1
<i>Vibrio bivalvicida</i>	VDREEEEAWEDELD	94	75.53%	WP_004745559.1
			74.47%	OAJ92073.1

FIGURE 2 | Homology comparison of *Vibrio parahaemolyticus* HY9901 T3SS Regulator Protein TyeA. *Vibrio alginolyticus* T3SS regulatory protein TyeA Accession No. ACY41068.1; *Vibrio parahaemolyticus* T3SS regulatory protein TyeA Accession No. ANB96904.1; *Vibrio harveyi* T3SS regulatory protein TyeA Accession No. SQA36766.1; *Vibrio campbellii* T3SS regulatory protein TyeA Accession No. AQM67418.1; *Vibrio tubiashii* T3SS regulatory protein TyeA Accession No. WP_004745559.1; *Vibrio bivalvicida* T3SS regulatory protein TyeA Accession No. OAJ92073.1.



with the highest homology shared with *V. parahaemolyticus* (83%). An untagged in-frame deletion mutant HY9901 Δ tyeA was constructed by over-lap PCR and forward and reverse screening methods. The mutant was confirmed by inability to grow on TSA supplemented with chloramphenicol, and verified by PCR by generating a fragment of ~1,092 bp. Using PCR the genome of HY9901 Δ tyeA and wild-type strain HY9901 by primer tyeA-up/tyeA-down, a 1,252 bp fragment was obtained for HY9901 Δ tyeA and fragment of 1,489 bp was obtained for HY9901 (Figure 3).

Characterization of the tyeA Genetic Stability of Mutants

After 30 generations of continuous passages of mutant Δ tyeA, the PCR genome of HY9901 Δ tyeA and wild-type strain HY9901 using primers tyeA-up/tyeA-down, a fragment of 1,489 bp was obtained for HY9901, and fragment of 1,252 bp was obtained for HY9901 Δ tyeA. This shows that the wild-type strain HY9901 has 237 bp more than the mutant Δ tyeA, and the mutant Δ tyeA has deleted the gene tyeA, which can stabilize the inheritance (Figure 4).

Comparison of Growth Rates of Wild Type and Mutant Bacteria

The growth rate of wild-type strain HY9901 and HY9901 Δ tyeA was similar and not statistically significant ($p > 0.05$) (Figure 5), the deletion of tyeA gene has no effect on the growth of *V. alginolyticus*. The exponential growth phase of the two bacterial strains was from 0 to 4 h, with growth stationary at 18 h, OD600 \approx 1.7.

Extracellular Protease Activity

Extracellular proteases, as metabolites of bacteria, play an important role as virulence factors in the process of infecting the host. Extracellular proteins have a variety of protease activities, including lecithin, amylase, lipase, and casein. The extracellular protease activity was not significantly different between HY9901 Δ tyeA and the wild-type strain HY9901 ($p > 0.05$) (Table 4).

Detection of Biofilm Formation Ability

No significant differences were observed in biofilm formation at 24 h ($p > 0.05$) between wild strains and mutants of *V. alginolyticus* using the crystal violet ammonium oxalate assay (Figure 6) or by confocal microscopy (Figure 7 and Table 4).

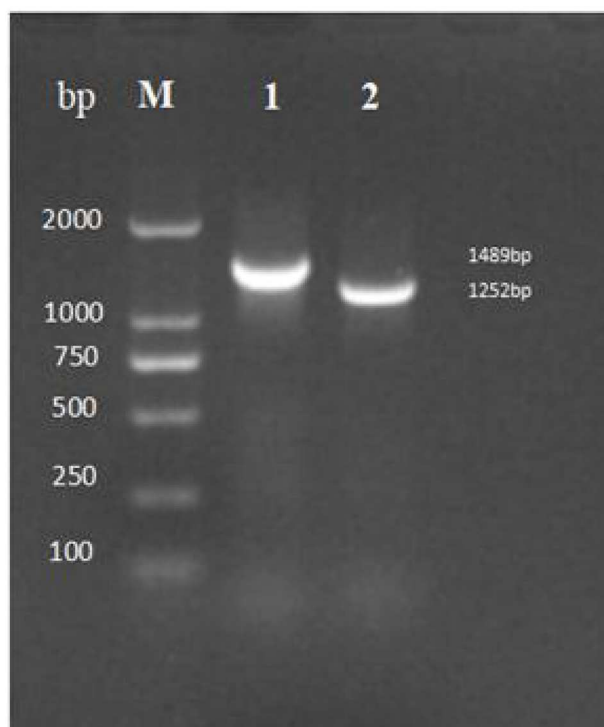


FIGURE 4 | Genetic stability detection of HY9901 Δ tyeA deletion mutant. M: DL2000 marker, Lane 1. A fragment of 1,489 bp is obtained for HY9901 using primer pairs of tyeA-up/tyeA-down. Lane 2. A fragment of 1,252 bp is obtained for HY9901 Δ tyeA using primer pairs of tyeA-up/tyeA-down.

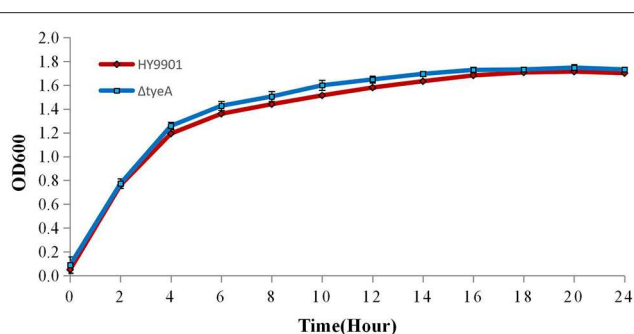


FIGURE 5 | Growth rates of HY9901 Δ tyeA and HY9901. Aliquots of cell culture were taken at various time points and measured for cell density at OD₆₀₀.

The biofilm fluctuates up and down within 72 h in crystal violet ammonium oxalate. The biofilm was thicker in HY9901 than in the mutant at 6 h ($p < 0.05$). After 12 h, the biofilm thickness was basically equal, and there was no significant change according to biological statistical analysis.

Swarming Motility

The wild-type strain HY9901 and HY9901 Δ tyeA were inoculated on the swarming plate, and the results were as follows: the swarming circle of wild strain was 34.9 ± 0.2 mm, mutant

TABLE 4 | Comparison of biological characteristics between HY9901 and HY9901 Δ tyeA.

Characteristics	HY9901	HY9901 Δ tyeA
Activity of ECPase(A ₄₂₂) ^a	1.03 ± 0.2	0.97 ± 0.2
Biofilm thickness(μ m) ^b	60 ± 10	90 ± 20
Swarming (mm) ^c	34.9 ± 0.2	$20.6 \pm 0.4^{**}$
LD ₅₀ (CFU mL ⁻¹) ^d	5.8×10^5	$2.6 \times 10^7^{**}$

Values are mean \pm standard deviation for three trials. Significant differences between HY9901 and HY9901 Δ tyeA indicated by asterisk.

^{**} $p < 0.01$.

^aBacteria were incubated in TSB for 18 h at 28°C.

^bBacteria were incubated in a glass bottom culture dish (NEST) for 24 h at 28°C.

^cSwarming diameters were measured after 24 h incubation on TSA containing 0.3% agar plates.

^dLD₅₀ were evaluated in healthy zebrafish with an average weight of 0.2 g.

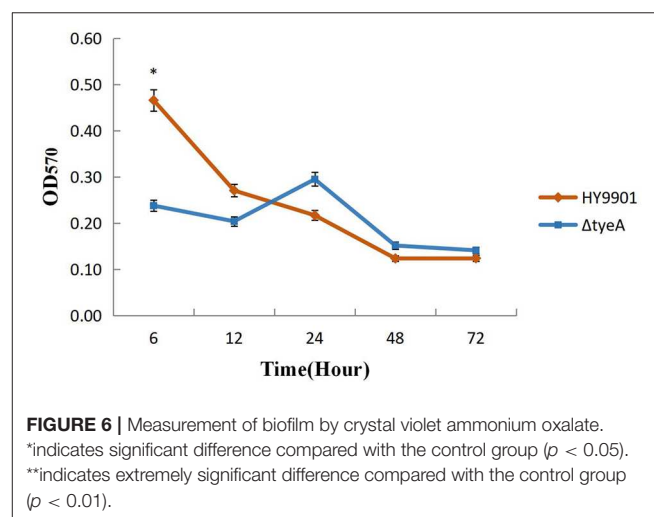


FIGURE 6 | Measurement of biofilm by crystal violet ammonium oxalate. *indicates significant difference compared with the control group ($p < 0.05$). **indicates extremely significant difference compared with the control group ($p < 0.01$).

was 20.6 ± 0.4 mm (Table 4). The swarming circle diameter of HY9901 Δ tyeA was significantly smaller than that of wild strain ($p < 0.01$), indicating that the swarming ability of HY9901 Δ tyeA was significantly weakened.

LD₅₀ Determination

Zebrafish from quarantined stocks recognized as disease free (Xu et al., 2010) were used as models (Sullivan and Kim, 2008) to assess the virulence of the strain HY9901 and HY9901 Δ tyeA. The results showed that the 50% lethal dose of HY9901 Δ tyeA was 40 times higher than that of wild strain (Tables 3, 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 tyeA was significantly decreased when compared with the wild strain ($p < 0.01$).

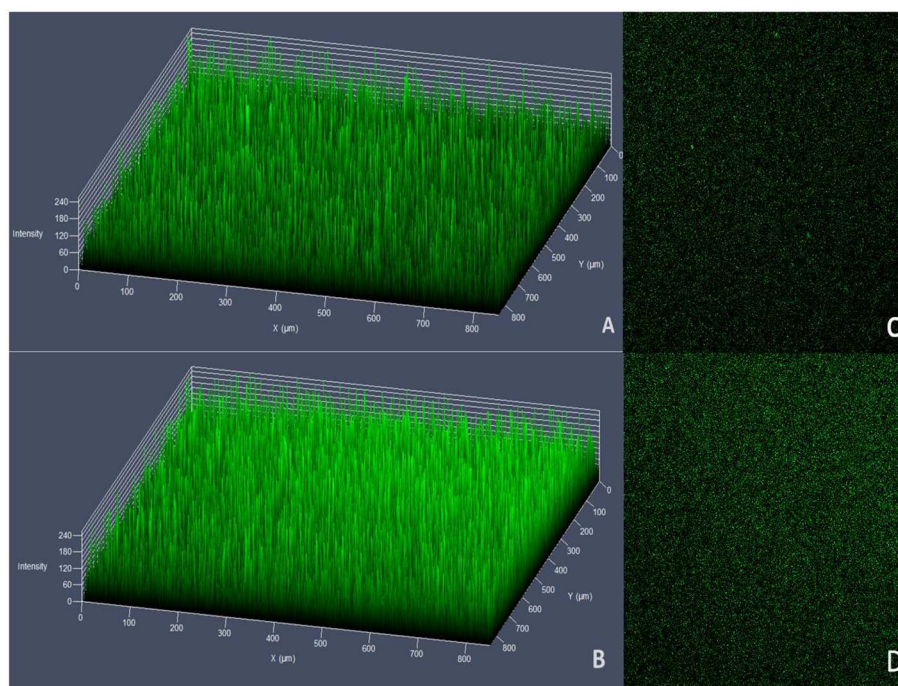


FIGURE 7 | Measurement of biofilm by LSCM. **(A)** HY9901 2.5d diagram. **(B)** HY9901 Δ tyeA 2.5d diagram. **(C)** HY9901 2d diagram. **(D)** HY9901 Δ tyeA 2d diagram; HY9901 Biofilm thickness: $60 \pm 10 \mu\text{m}$, HY9901 Δ tyeA Biofilm thickness: $90 \pm 20 \mu\text{m}$.

Antibiotic Susceptibility

Both wild and mutant strains were extremely susceptible to amikacin, minocycline, gentamicin, Cefperazone; resistant to oxacillin, clindamycin, ceftazidime, penicillin, ampicillin, caeбенicillin, cefazolin, ceftriaxone, cephradine, piperacillin. Wild strains were sensitive to tetracycline, chloramphenicol, kanamycin, doxycycline, while mutant strains were resistant to them (Table 5).

T3SS-related Gene Expression Analysis

QRT-PCR was employed to analyze the transcription levels of T3SS-related genes including *vscl*, *vsck*, *vopN*, *vsco*, *vsclN*, *vopS*, and *hop*. The results showed that compared with HY9901 wild type, Δ tyeA had significantly increased expression of *vscl*, *vsck*, *vsco*, *vopS* ($p < 0.05$), *vopN*, *vsclN* and *hop* ($p < 0.01$) (Figure 8).

Vaccine Efficacy

Fish vaccinated with the mutant strain HY9901 Δ tyeA were challenged 28 days post-vaccination with the wild strain. Within 14 days, the mortality rate in the control group injected with sterile PBS was 87%, the mortality rate in the injection immunization group was 25%, and the relative percentage survival was 71.2% (Figure 9).

Immune Gene Expression of Zebrafish Induced by HY9901 Δ tyeA Vaccine

Detection of immune gene expression in zebrafish immunized with HY9901 Δ tyeA 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 the mutant strain HY9901 Δ tyeA had significantly increased expression of IL6, IL6R, IL-1 β , TNF- α , rag-1, TLR5, c/ebp β genes in liver and IL6, IL8, IgM, TNF- α , rag-1 genes in spleen compared to control fish injected with PBS ($p < 0.01$) (Figure 10).

DISCUSSION

At present, the interaction mechanisms between some fish pathogens and hosts have been studied in depth. Based on these studies, the development of efficient new vaccines has become highly desirable in aquaculture (Pang et al., 2016). Attenuated live vaccines are one of the more efficient vaccines, especially compared to inactivated vaccines due to their greater activation of cellular immunity. Currently, the construction of attenuated strains by gene knockout is an important method to obtain candidate attenuated live vaccines.

In the current study, we knocked out the T3SS gene *tyeA* of *V. alginolyticus*, explored its biology and pathogenicity, and evaluated its effect as a live attenuated vaccine. Zhou et al. (2013) found that the mutant of T3SS chaperone escort *vsco* of *V. alginolyticus* showed an attenuated swarming ability and a 10-fold decrease in the virulence to fish. However, the Δ vsco mutant showed no difference in the biofilm formation and ECPase activity. The authors concluded that *vsco* is associated with the flagella of *V. alginolyticus*. The knockout of *vsco* gene results in the decline of group movement ability and thus reduced virulence

TABLE 5 | Drug sensitivity test results of the HY9901 and HY9901 Δ tyeA.

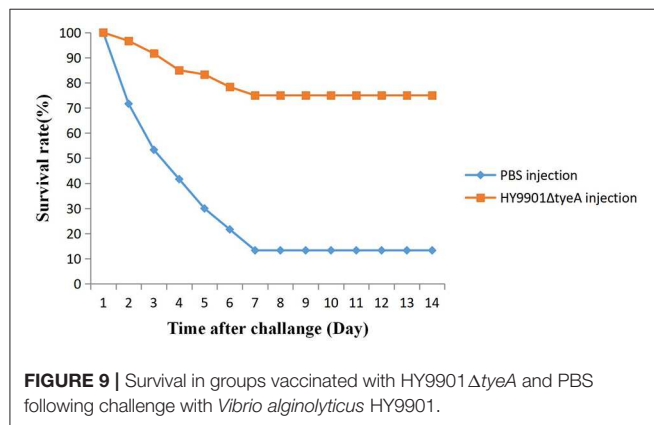
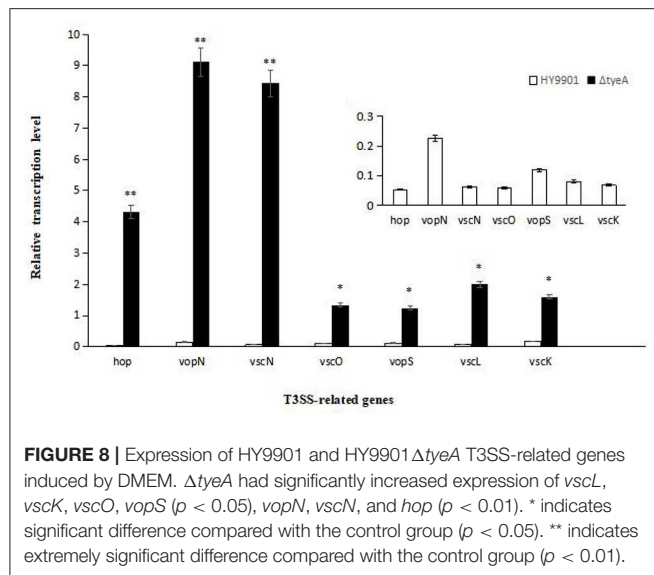
Antibiotic	Dose (μ g)	Bacteriostatic circle diameter (mm)			
		HY9901	Sensitivity	Δ tyeA	Sensitivity
Cefperazone	75	0	R	0	R
Oxacillin	1	0	R	0	R
Clindamycin	2	0	R	0	R
Ceftazidime	30	0	R	0	R
Penicillin	10U	0	R	0	R
Ampicillin	100	0	R	0	R
Caebenicillin	100	0	R	0	R
Cefazolin	30	8.0 \pm 0.2	R	0	R
Ceftriaxone	30	9.3 \pm 0.3	R	0	R
Cephadrine	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	0	R
Vancomycin	30	0	R	0	R
Cephalexin	30	0	R	0	R
polymxinB	200IU	0	R	0	R
Norfloxacin	10	0	R	0	R
Ofloxacin	5	0	R	0	R
Ciprofloxacin	5	0	R	0	R
Amikacin	30	13.3 \pm 0.3	I	10.0 \pm 0.1	I
Minocycline	30	18.5 \pm 0.2	S	14.3 \pm 0.3	I
Tetracycline	30	13.5 \pm 0.2	I	12.5 \pm 0.2	R
Gentamicin	10	14.5 \pm 0.1	I	12.5 \pm 0.2	I
Furazolidone	300	10.5 \pm 0.4	R	8.0 \pm 0.1	R
Chloramphenicol	30	17.2 \pm 0.3	S	0	R
kanamycin	30	14.1 \pm 0.2	I	12.1 \pm 0.2	R
Erythromycin	15	10.1 \pm 0.2	R	0	R
Doxycycline	30	16.5 \pm 0.3	S	10.6 \pm 0.1	R
Cefperazone	30	14.2 \pm 0.3	I	13.3 \pm 0.3	I

S(susceptible) I(intermediate) R(resistance).

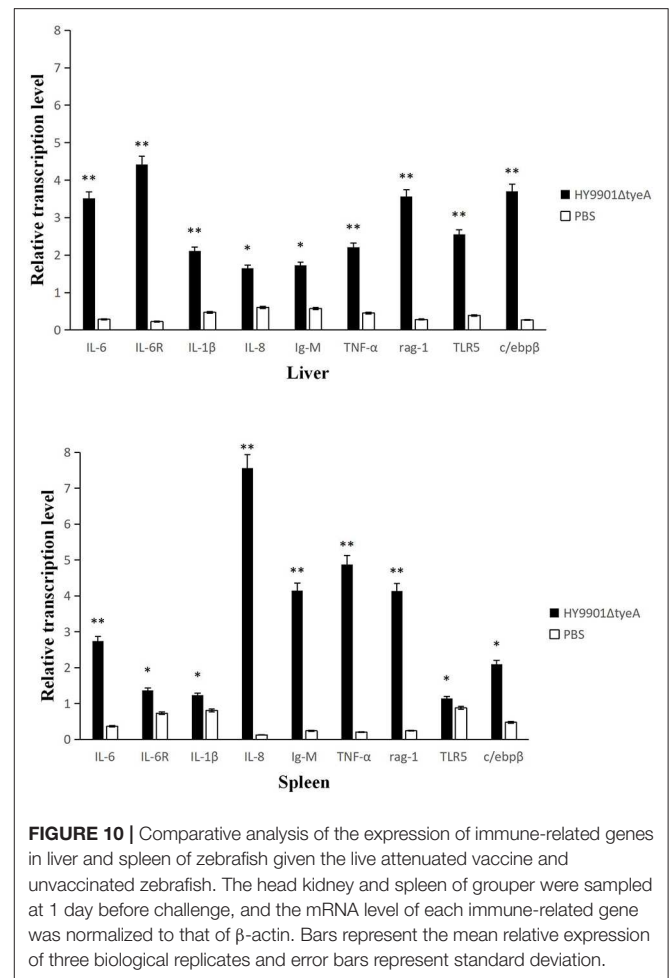
(Zhou et al., 2013). Another mutant of *V. alginolyticus*, Δ sodB did not show any difference in growth when compared with wild type strains HY9901. However, the Δ sodB mutant resulted in the formation of biofilm, increased SOD activity and toxicity, increased ECPase activity and sensitivity to hydrogen peroxide, a decreased group movement ability and decreased adhesion to cytokine-induced killer (CIK) cells (Yanyan et al., 2019). Even though the mutant of T3SS effector Hop revealed an attenuated swarming phenotype and a 2,600-fold decrease in the virulence

to grouper, the HY9901 Δ hop mutant showed no difference in morphology, growth, biofilm formation and ECPase activity (Pang et al., 2018). Similarly in the present study, there was no significant difference between HY9901 Δ tyeA and wild type strains HY9901 in growth, biofilm formation and ECPase activity but the HY9901 Δ tyeA mutant showed an attenuated swarming phenotype and a nearly 40-fold decrease in virulence to zebrafish. A polar flagellum and numerous lateral flagella contribute to the swimming and swarming motilities respectively, by which some bacteria access an appropriate niche inside the host after infection, as observed in *V. parahaemolyticus* (Enos-Berlage et al., 2005). Therefore, swarming ability is closely related to the virulence of some bacteria. Biofilm formation is a multicellular behavior through which bacteria colonize the surface of host tissues and tank surfaces and are thereby highly resistant to antibiotics and host immune defense mechanisms (Parsek and Singh, 2003; Verstraeten et al., 2008). Extracellular products (ECP) produced by bacteria include proteases, hemolysins and siderophores. As a metabolite of bacteria, extracellular proteases used as virulence factors by *V. alginolyticus* play an important role in the process of infection. Flagella assist in swimming and can help bacteria enter the appropriate niche in the host after *Vibrio* spp. infection. Motility is an important index used to judge the virulence of bacteria (Watnick et al., 2001). Numerous studies have shown that flagellin is essential for flagellum formation, trivial movement and symbiosis, and it also greatly affects the colonizing ability of *Vibrio* spp. (Millikan and Ruby, 2004). The results of this study showed that there was no significant difference in bacterial growth rate, biofilm, and extracellular enzyme activity between the *tyeA* knockout and the wild strain of *V. alginolyticus*, suggesting that TyeA may not affect these characteristics in *V. alginolyticus*. Nevertheless, this study found that HY9901 Δ tyeA had significantly decreased swarming motility, which may be associated with bacterial flagellar movement. Therefore, the *tyeA* gene may have a positive regulation in the swarming motility of *Vibrio alginolyticus*, and its regulatory mechanism needs further investigation.

It is generally agreed that T3SS can be secreted by bacteria *in vitro* or by contacting host cells (Buttner and Bonas, 2002; Blondel Carlos et al., 2016). The secretory pathway of T3SS *in vitro* and *in vivo* is complex, sometimes the regulation is completed by a single regulatory protein, and sometimes the regulation is completed by multiple interacting regulatory proteins. For example, the transcriptional expression of the T3SS1 gene cluster of *V. parahaemolyticus* in DMEM can be regulated by the regulatory proteins ExsA and ExsD. Among them, ExsA is a positive transcriptional regulation protein and ExsD is a negative transcriptional regulation protein. It was hypothesized that ExsA might play a direct role in the T3SS1 promoter sequence (Zhou et al., 2008) based on the discovery that ExsA protein directly binds the upstream region of effector protein VP1668 and VP1687 in gel retardation assay (EMSA). Furthermore, the co-expression of recombinant proteins labeled with different antigens showed that: the combination of ExsD and ExsA blocks the expression of T3SS1; the combination of ExsC and ExsD releases ExsA, thus allowing the expression of T3SS1 (Zhou et al., 2010).



According to Miller et al. (2016), in a bile salt environment, VttRA and VttRB of *V. cholerae* can regulate the secretion of T3SS structural genes and some effector proteins. In terms of *V. alginolyticus*, ExsA and ExsC play a positive regulatory role on the effector proteins Va1686 and Va1687, ExsD, and ExsE play a negative regulatory role, ExsA and ExsC act as stimulus to the swarming of the side flagella of *V. alginolyticus* and on the contrary, ExsD and ExsE act as hindrance to the swarming of the side flagella (Liu et al., 2016). Interestingly, the results of fluorescence quantification and drug susceptibility tests in this study indicated that TyeA negatively regulated T3SS protein (vscL, vscK and vscO are apparatus proteins, while vopN, vscN, and hop are effector or regulatory proteins) and drug resistance genes. However, the systematic research of the network regulatory mechanism of various *V. alginolyticus* regulatory proteins on effector proteins *in vitro* and *in vivo* is still needed. TyeA, a regulator in T3SS, is widely studied in other strains (Sundberg and Forsberg, 2003; Schubot et al., 2004; Amer et al., 2016), and some progress has been made (Joseph and Plano, 2007; Plano and Schesser, 2013). However,



its regulation mechanism in *V. alginolyticus* remains unknown. In order to further reveal the pathogenic mechanism of *V. alginolyticus*, so as to achieve the purpose of controlling vibriosis, the research on the regulatory mechanism of the regulatory gene *tyeA* in *V. alginolyticus* will be followed-up in future investigations.

Moreover, although zebrafish will not specifically be the target of the vaccine in the future, studies have shown that zebrafish are a good model for teleost immune responses (Streisinger et al., 1981). For example, Zhang et al. (2012) used zebrafish to investigate the immune response following administration of a live attenuated *V. anguillarum* vaccine. Hua and co-author's results showed that Th17 cells were activated following vaccination of zebrafish (Hua et al., 2013). At the same time, a large number of studies have shown that zebrafish can be infected by marine vibrios (O'toole et al., 2004; Rojo et al., 2007; Rodríguez et al., 2008). Therefore, it is feasible to use zebrafish to study the effectiveness of a marine *Vibrio* vaccine. In this study, genes associated with innate immunity or inflammatory factors: IL-1 β, IL6, IL8, and TNF- α were studied. Additionally the expression profiles of bacterial flagellum recognition factor TLR5, adaptive immune factors including

rag-1, and immunoglobulin IgM were assessed, as well as blood corpuscle specific marker genes *c/ebp*. In summary, the results show that HY9901 Δ *tyeA* can effectively induce the protective immune response of zebrafish associated with pro-inflammatory and immunoglobulin activity. *V. alginolyticus* has been resistant to most antibiotics according to the results of drug sensitivity testing in this study. Antibiotic resistance is closely linked to antibiotic abuse (Chee-Sanford et al., 2001; BurrIDGE et al., 2010). In the future, the direction for the prevention and control of aquatic diseases such as Vibriosis is to develop *Vibrio* vaccine and thereby reduce the use of antibiotics, which has great significance for protecting the environment and developing aquaculture sustainably. In the current study, the relative percentage survival rate of zebrafish immunized with the vaccine candidate strain Δ *tyeA* was 71.2%, which indicated that the construction of the deletion strain could be used as one of the effective ways to develop a vaccine against this pathogen. It provides an experimental basis for the prevention and control of *Vibrio* spp. in aquaculture. Studies have shown that compared with inactivated vaccines, live attenuated vaccines have the advantages of long-lasting efficacy and inducing strong cellular immunity, which is in line with the current needs of aquaculture, however their use is not permitted under current regulations in Europe.

CONCLUSION

To sum up, we have successfully constructed the deletion strain of HY9901 Δ *tyeA*. We found that the HY9901 Δ *tyeA* strain exhibited decreased virulence to zebrafish, but the wild strain and the mutant strains were resistant to most antibiotics. The attenuated live vaccine was highly protective against *V. alginolyticus* and can induce protective immune responses in zebrafish. These results provide further evidence for the importance of T3SS in *V. alginolyticus* and provide reference for further study of this virulence factor. The

immune effect and safety of the attenuated live vaccine will be tested and evaluated in aquaculture species in order to provide further theoretical basis and technical support for its application for the prevention and control of fish diseases caused by *V. alginolyticus*.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the NCBI Accession No. MN328351.

ETHICS STATEMENT

The animal study was reviewed and approved by Guangli Li and Guangdong Ocean University of ethics committee.

AUTHOR CONTRIBUTIONS

SZ and HP designed the experiments. SZ, XT, and JL generated experimental data and wrote the manuscript. HP, RH, SM, and JJ conceived the work and critically reviewed the manuscript. All authors contributed extensively to the work presented in this manuscript.

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Conflict 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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