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Characterization of a Diguanylate Cyclase VAGM001033 of *Vibrio alginolyticus* and Protective Efficacy as a Live Attenuated Vaccine Candidate in Pearl Gentian Grouper

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Vibrio alginolyticus (*V. alginolyticus*) is one of the important epizootic pathogens in marine animals. VAGM001033 belongs to a diguanylate cyclase, responsible for the synthesis of dimeric guanosine monophosphate (c-di-GMP), a ubiquitous second messenger involved in the function of biofilm formation, motility, and virulence. This study confirmed that VAGM001033 was an active diguanylate cyclase by Congo red assay. The red-stained, dry, and rough form of colonies were observed with the increasing concentration of the L-arabinose on Congo red plates. Also, an in-frame deleted Δ*VAGM001033* mutant was constructed and changes of Δ*VAGM001033* mutant in physiology and pathogenicity were detected. The Δ*VAGM001033* mutant displayed similar morphology and growth curve with the wild-type strain showing no significant differences. The swarming ability of the Δ*VAGM001033* mutant was significantly enhanced showing bigger swarming circles, while the biofilm formation, extracellular proteases, and virulence were significantly attenuated. The results of the test for antibiotic susceptibility showed that the wild type and Δ*VAGM001033* mutant had similar sensitivity or resistance to most antibiotics used in this study, except cefotaxime and nitrofurantoin. The mutant was sensitive to cefotaxime and nitrofurantoin, while the wild type was intermediate. A total of 756 differentially expressed genes (DEGs) were identified by RNA-seq, of which 109 were upregulated and 647 were downregulated. Flagellar assembly, two-component system, ATP-binding cassette (ABC) transporters, and peptidoglycan biosynthesis were significantly enriched in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway. Moreover, the Δ*VAGM001033* mutant induced high antibody titers and provided immune protectivity with a relative percent survival (RPS) of 82%. Immune-related genes of pearl gentian grouper (♀*Epinephelus*

fuscoguttatus × ♂*Epinephelus lanceolatus*), namely, *IgM*, *MHC-Iα*, interleukin-1β (*IL-1β*), interleukin-16 (*IL-16*), and tumor necrosis factor-α (*TNF-α*) were upregulated after vaccination. Overall, the results suggested that VAGM001033 plays a crucial role in *V. alginolyticus*. The Δ*VAGM001033* mutant might be applied as an effective live vaccine candidate against *V. alginolyticus*.

Keywords: *Vibrio alginolyticus*, *VAGM001033*, diguanylate cyclase, live attenuated vaccine, mutant

INTRODUCTION

Vibrio alginolyticus (*V. alginolyticus*), a Gram-negative bacterium, is one of the important epizootic pathogens to marine animals. Despite the boom of fish farming in China, the farmed-marine industry has been severely hampered by fish mortality due to vibriosis diseases caused by *V. alginolyticus* (Lee et al., 2020). In July of 2017, there was an outbreak of vibriosis among seahorses, featured by severe ulcers on the skin, caused by the dominant causative species *V. alginolyticus* in Eastern China (Xie et al., 2020). Cai et al. (2018) stated that it is considered to be the major causative media of outbreak of vibriosis in fish and shellfish at the coast of South China in the recent years. *V. alginolyticus* infection caused tissue necrosis, skin ulceration, gastroenteritis, and inflammatory reactions in aquatic animals (Chen et al., 2020; Amenyogbe et al., 2021; Su et al., 2021).

GGDEF domain protein is a cyclic di-guanosine monophosphate (c-di-GMP)-specific diguanylate cyclase, which is responsible for the synthesis of c-di-GMP. A plethora of studies confirmed that GGDEF domain protein was critical for enzyme activity, which regulated many functions, namely, motility (Floyd et al., 2020; Webster et al., 2021), biofilm formation (Valentini and Filloux, 2019), and virulence (Joshi et al., 2020). The first GGDEF domain protein PleD was identified in *Caulobacter cresenctus*, which controlled the cell transition from swarms to stalks (Hecht and Newton, 1995). WspR was verified to contain a GGDEF domain, which controlled the autoaggregation in *Pseudomonas aeruginosa* (Hueso-Gil et al., 2020). FimX was reported to bear a GGDEF domain, which regulated the twitching motility in *Pseudomonas aeruginosa* (Jain et al., 2017). RocS was also reported to regulate the motility in *Vibrio cholerae*, which included a GGEDF domain (Wu et al., 2020). ScrC in *Vibrio parahaemolyticus* was reported to regulate the cell attachment and motility for containing a GGEDF domain (Kimbrough and McCarter, 2020). The gene, referred to in the article as *VAGM001033*, consisted of a 1,035 bp open reading frame (ORF) that encoded 344 amino acids contained a predicted GGDEF domain. However, the enzymatic activity and regulatory mechanisms of these kinds of enzymes are still relatively scarce in *V. alginolyticus*.

With the expansion of the fish breeding scale, increased emergence and outbreak of fish diseases posed harm to the aquaculture industry. Antibiotic abuse led to the appearance of drug-resistant strains and food safety (Gudding and Van Muiswinkel, 2013). Hence, seeking a safe and effective solution is particularly urgent in aquaculture. Vaccination is definitely the most appropriate method to prevent and control fish diseases. The earliest bony vertebrate with both innate and adaptive immune response is reported to be teleost fish (Cooper and Alder, 2006).

For the purpose to detect the role of *VAGM001033* in *V. alginolyticus*, the Δ*VAGM001033* mutant was constructed and some physiological characteristics of Δ*VAGM001033* mutant were detected in this study. Furthermore, pearl gentian grouper was used to evaluate the efficacy of the mutant strain as an attenuated live vaccine candidate that will contribute to the healthy and sustainable development of the aquaculture industry.

MATERIALS AND METHODS

Bacterial Strains and Fish

In this study, the bacteria, plasmids, and primers were listed in **Table 1**. *V. alginolyticus* strain HY9901 was separated from a sick maricultured grouper (*Epinephelus coioides*) at Zhanjiang, China, and stored in our laboratory. *V. alginolyticus* was grown at 28°C on Tryptic soya broth (TSB) medium supplemented with 2% NaCl (Haling, China). *Escherichia coli* (*E. coli*) strains were cultured at 37°C in Luria broth (LB) (Haling, China). Ampicillin (Amp, 100 μg/ml) or chloramphenicol (Cm, 25 μg/ml) were supplemented when the optimal antibiotics were needed.

Pearl gentian groupers (♀*Epinephelus fuscoguttatus* × ♂*Epinephelus lanceolate*, 30.0 ± 3.0 g) were purchased from a local fish farm from Donghai island, Zhanjiang and maintained in seawater at a temperature of 28 ± 1°C in a recirculation system for 2 weeks. Fishes were considered healthy by bacteriological recovery tests as described previously (Pang et al., 2022).

Before the extraction of tissues after the cessation of the experiment, fishes were anesthetized with MS222 (tricaine methanesulfonate, 100 ng/ml). All the fish experiments were approved by the respective Animal Research and Ethics Committees of the Guangdong Provincial Key Laboratory of Pathogenic Biology and Epidemiology for Aquatic Economic Animals.

Congo Red Assay

A pair of primers pBAD-*VAGM001033*-F/pBAD-*VAGM001033*-R were designed as shown in **Table 1** according to *V. alginolyticus* genomic sequences (GenBank number: CP072781-CP072782). PCR has conducted following the conditions: denaturation at 95°C for 5 min, followed by 38 cycles of 94°C for 30 s, 55°C for 60 s, and 72°C for 30 s in a Thermocycler (Bio-Rad, Hercules, CA, United States). The PCR product was examined on 1% agarose gel, then was purified and ligated into the

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

Strains, plasmids, and primers	Relevant information or sequence (5′-3′)	Sources
Vibrio alginolyticus HY9901	Wild-type strain	Pang et al., 2022
Δ*VAGM001033*	*V. alginolyticus* HY9901, in-frame deletion	This study
Escherichia coli DH5α	Competent cells	TakaRa
*Escherichia coli*β2163	Competent cells	Maibo Bioscience
pLP12	*E. coli* suicide vector	This study
pBAD-HisA	Amp^+	TakaRa
pBAD-*VAGM001033*-F	TAACCATGGGGATGCTGCTGGTGGTGTTTG	This study
pBAD-*VAGM001033*-R	TTCGAATTCTTAACGTTGAAATTTCATTTTGTAC	This study
pBAD-TF	CAAAGGGTCACTTAGCGCC	
pBAD-TR	CACTTCTGAGTTCGGCATGG	
VAGM001033-UF	GGAATCTAGACCTTGAGTCGTTTGTTGCGCCATTCTTGTCT	This study
VAGM001033-UR	GCGCTTATCCGCTATGCTGATCAAAGAAACGGCAAACACCAC	This study
VAGM001033-DF	GTGGTGTTTGCCGTTTCTTTGATCAGCATAGCGGATAAGCGC	This study
VAGM001033-DR	ACAGCTAGCGACGATATGTCTGTGGTGGGAGGGGGTAGTAA	This study
VAGM001033-TF	AACCTTGACGTGACGTAAACCGAC	This study
VAGM001033-TR	CGCTCATTCTTTCGATGTTATTTT	This study
IL-1βF	TCTGGGCATCAAGGGCACACA	Zhou et al., 2020
IL-1βR	CCATGTCGCTGTTCGGATCGA	
TNF-αF	GCCACAGGATCTGGCGCTACTC	
TNF-αR	CTTCCGTCGCTGTCCTCATGTG	
IL-16F	TTCAGATCCTCCGTCCAAC	
IL-16R	TCTGTTCTGCGGGTTTAGC	
IgM-F	TACAGCCTCTGGATTAGACATTAG	
IgM-R	CTGCTGTCTGCTGTTGTCTGTGGAG	
MHC-IαF	GCCGCCACGCTACAGGTTTCTA	
MHC-IαR	TCCATCGTGGTTGGGGATGATC	
16s-F	TTGCGAGAGTGAGCGAATCC	
16s-R	ATGGTGTGACGGGGCGGTGTG	
β-actinF	GGACAGCTACGTTGGTGATGA	
β-actinR	TGGTCACAATACCGTGCTCAATG	

pBAD-HisA vector. The recombinant plasmid was transformed into *E. coli* DH5α and subsequently *E. coli* BL21. The inserted fragment was sequenced by Sangon Biological Engineering Technology & Services Corporation, Ltd. (Guangzhou, China) using the primers pBAD-TF/pBAD-TR. Finally, the recombinant pBAD-*VAGM001033* was inoculated in Congolese red LB agar plates containing different concentrations of inducing agent L-arabinose. The assay was conducted in triplicates.

Construction of a Δ*VAGM001033* Mutant

The Δ*VAGM001033* mutant was constructed by homologous recombination techniques described by Chen et al. (2019b). Briefly, the upstream homologous fragment of 524 bp was amplified using a primer (primers: *VAGM001033*-UF and *VAGM001033*-UR) and the downstream homologous fragment of 493 bp was amplified using a primer (primers: *VAGM001033*-DF and *VAGM001033*-DR) from *V. alginolyticus* HY9901 genomic DNA. Both fragments contained a 20 bp overlapping sequence were used to generate an in-frame deletion of the *VAGM001033* gene by overlap extension PCR. Then, the overlap PCR product was ligated into a suicide T-vector plP12 (Luo et al., 2015), a universal genetic tool for rapid and efficient deleting mutation in Vibrio species, which is based on carrying a novel counterselectable *vmi480* marker. The recombinant plP12-Δ*VAGM001033* was transformed into *E. coli* DH5α and subsequently transformed into *E. coli* β2163. After homologous recombination two times, positive clones were selected and used for the conjugation with *V. alginolyticus*, and Δ*VAGM001033* mutant was successfully constructed using a PCR identification (primers: *VAGM001033*-TF and *VA001033*-TR).

Characterization of Δ*VAGM001033* Mutant

Cell Morphology

Overnight cultures were grown to the exponential phase in fresh medium. *V. alginolyticus* HY9901 strain and Δ*VAGM001033* mutant were prepared for scanning electron microscope as the method described by Wang et al. (2007). The samples were viewed and photographed using a Hitachi XA-650 scanning electron microscope (Hitachi, Japan).

Genetic Stability of the Δ*VAGM001033* Mutant

Δ*VAGM001033* mutant was inoculated onto a TSA plate and passed blindly for 30 generations according to the method

described by Zhou et al. (2020). In brief, a single colony was picked from Tryptic soya agar (TSA) plates and cultured in TSB (HKM, Guangzhou, China) with shaking for 12 h, and then bacteria broth culture was streaked out and cultured on a TSA plate. This process was repeated 30 times. The genetic stability of each generation was determined by PCR.

Growth Curve of Bacteria

Vibrio alginolyticus HY9901 and Δ*VAGM001033* mutant were cultured in TSB overnight. The culture strains were inoculated into TSB with an initial OD_{600} of 0.01, respectively. Samples were measured at OD_{600} every hour. This procedure was repeated three times in each group.

Swarming Motility

Swarming motility was performed according to the approach explained by Mathew et al. (2001). Briefly, single colonies of *V. alginolyticus* HY9901 and Δ*VAGM001033* mutant were selected and inoculated into TSA plates with 0.3% concentration of agar at 28°C, and swarming circle diameter was measured by Vernier calipers after 24 h incubation. The experiment was performed in triplicate.

Extracellular Protease Activity

Extracellular protease (ECPase) activity was performed as previously described by Windle and Kelleher (1997). *V. alginolyticus* HY9901 and Δ*VAGM001033* mutant were cultured on TSA plates coated with sterile cellophane at 28°C for 24 h, washed with sterile phosphate-buffered saline (PBS), centrifuged at 4°C for 30 min, and the supernatant filtered to collect extracellular products. Inactivated sample (supernatant was boiled for 10 min) was used as a blank control. The experiment was performed in triplicate.

Biofilm Formation

Biofilm formation was assayed using crystal violet ammonium oxalate concerning the method described by Kierek and Watnick (2003). A total of 200 μl bacteria suspension (OD_{600} = 0.5) was inoculated in a 96-well plate with 3 replicates per sample at 28°C. Samples were taken at 24 h, fixed for 20 min with methanol, and stained with crystal violet ammonium oxalate dye for 15 min. Finally, 95% alcohol was then added and incubated at room temperature for 30 min. OD_{570} was determined by using a Multimode Plate Reader (PerkinElmer EnSpire, Singapore). The experiment was performed in triplicate.

LD_{50} Determination

The injection concentration of *V. alginolyticus* HY9901 and Δ*VAGM001033* mutant were 10^5, 10^6, 10^7, 10^8, and 10^9 colony-forming unit (CFU)/ml. A total of 330 pearl gentian groupers were randomly divided into three groups. In the *V. alginolyticus* HY9901 group and the Δ*VAGM001033* mutant group, 100 μl bacterial suspension was intraperitoneally injected into each fish. In the PBS control group, all the fishes were injected with 100 μl PBS in the same manner. Mortalities of fish were recorded over 14 days until the mortality rate was stable. The mortality challenged by *V. alginolyticus* was determined and the LD_{50} value of the wild-type strain and Δ*VAGM001033* mutant were calculated according to the modified Coriolis method (Cai et al., 2018). The experiments were conducted in triplicate.

Antimicrobial Susceptibility Test

The antimicrobial susceptibility tests were conducted using the Kirby–Bauer disc diffusion method (Bauer, 1966) on TSA nutrient agar plates according to Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute [CLSI], 2013) and also following manufacturer's guidelines. The antibiotic impregnated discs were purchased from Binhe Microbial Reagent Corporation, Ltd., Hangzhou, China, namely, polymyxin B, cefoxitin, cefuroxime, kanamycin, oxacillin, gentamicin, vancomycin, cefuroxime, amikacin, cefoperazone, spectinomycin, ceftriaxone, chloramphenicol, SMZ/TMP, clarithromycin, tetracycline, norfloxacin, ofloxacin, cefixime, cefotaxime, and nitrofurantoin. Vernier calipers were used to scale the diameters of the inhibition zones. The experiments were conducted in triplicate.

Transcriptome Sequencing

For transcriptome sequencing, *V. alginolyticus* HY9901 and Δ*VAGM003125* mutant were cultured in TSB media at 28°C overnight. According to the method of Pang et al. (2022), the bacterial cells were harvested and dissolved in TRIzol (Takara). The followed experiments, namely, RNA extraction, RNA fragmentation, cDNA synthesis, RNA-Seq libraries construction, and bioinformatics analysis were conducted by Gene Denovo Biotechnology Corporation, Ltd. (Guangzhou, China). The results of the transcriptome sequencing have been deposited at the SRA database under accession numbers PRJNA794348.

Vaccination and Bacterial Challenge

Vaccination experiments were performed to evaluate the immunoprotectivity of Δ*VAGM001033* mutant as a live attenuated vaccine candidate against *V. alginolyticus* as the previous study of Chen et al. (2019c). Fishes were randomly divided into two groups with 90 fishes per group. Fishes in the Δ*VAGM001033* mutant group were injected intraperitoneally with 100 μl 1.0×10^5 CFU/ml Δ*VAGM001033* mutant. Control fishes were injected intraperitoneally with 100 μl sterile PBS. The experiment was conducted in triplicates.

Six weeks post-immunization, fishes (n = 30) were anesthetized and challenged separately by 100 μl 1×10^8 CFU/ml of *V. alginolyticus* HY9901 in each group. The cumulative mortality was recorded for 14 days after the challenge. The relative percent survival (RPS) was calculated according to the formula: RPS = 100% [1 – (mortality of the Δ*VAGM001033* mutant group/mortality of the PBS control group)]. The bacteria were reisolated from the liver and spleen of all the dead fishes and identified by 16s rDNA. The experiment was conducted in triplicates.

Analysis of Antibody Levels

During the experimental period from 1 to 6 weeks post-vaccination, approximately 200 μl of blood from individual fish (3 fish per group) in the PBS group and the Δ*VAGM001033*

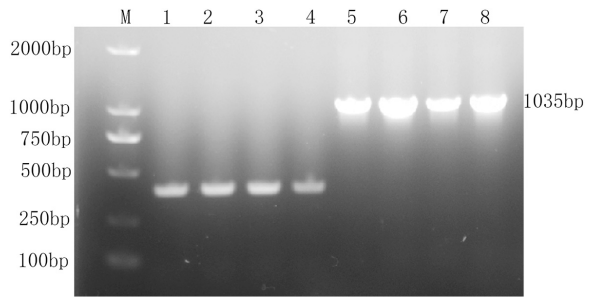

FIGURE 1 | Construction of pBAD-*VAGM001033*. M: DL 2,000 marker; lane 1–4: pBAD-HisA; lane 5–8: pBAD-HisA-*VAGM001033*.

mutant group are collected. Serum was collected following centrifugation at 15,000 g for 5 min to measure antibody levels using ELISA as the method described by Chen et al. (2019b). Briefly, microtiter plate wells were coated with 100 μl of formalin-killed *V. alginolyticus* by overnight incubation at 4°C. Excess cells were discarded and wells were blocked with 100 μl of PBS containing 2% bovine serum albumin (BSA) for 3 h at 22°C. After removing the blocking solution and washing three times with PBS added with 0.05% Tween-20 (PBST), the wells were incubated for 3 h at 22°C with 100 μl of serially diluted fish serum. Antibody binding to the antigen was detected using mouse antipearle gentian grouper IgM polyclonal antibody (1:10,000), followed by rabbit antimouse IgG-HRP (Wuhan Boster, Wuhan, China) at 1:20,000 dilutions, and color was developed with a chromogenic reagent tetramethylbenzidine (TMB) (Ameresco, Ltd., Framingham, MA, United States) for 20 min with the reaction being stopped by the addition of 2.0 M H_2SO_4. The plates were then read at 450 nm with a microplate reader (Bio-Rad). The experiment was conducted in triplicates.

Histopathological Analysis

After 42 days post-immunization, liver and intestine tissues (3 fish per group) were taken from the PBS group, the Δ*VAGM001033* mutant group, and the *V. alginolyticus* HY9901 group. According to the previous study (Xie et al., 2020), the tissues were fixed in Davidson's fixative (Shanghai Tarui Bioscience, China) at room temperature for 24 h. Tissues were dehydrated with a series of different concentrations of ethanol, cleared in xylene, and embedded into paraffin. Paraffin blocks of tissues were cut at 5 μm thickness on decontaminated glass slides. After deparaffinization, samples were stained with H&E and observed under an optical microscope (Leica, Germany).

Expression Analysis of Immune-Related Gene of Grouper

According to the method described by Wei et al. (2020), liver, spleen, head, and kidney (3 fishes per group) were taken from individual fish in the PBS group and the Δ*VAGM001033* mutant group at 42 days post-immunization. The real-time quantitative PCR (qPCR) was performed according to standard protocols with the use of the SYBR Green qPCR Supermix Kit (TransGen, China). The final reaction volume of 20 μl contained 1 μl cDNA, 10 μl of 2 × SYBR Green qPCR SuperMix, 1 μl of each primer (10 μM), and 7 μl of ddH_2O. The reaction was performed under the following conditions: 95°C 30 s; 40 cycles for 95°C 15 s, 55°C 20 s, and 72°C 30 s in LightCycler®96 (Roche, United States). A melting curve analysis was performed to access the amplification of specific products. The primers for interleukin-16 (*IL-16*), *IL-1*, *IgM*, *MHC-Iα*, and tumor necrosis factor-α (*TNF-α*) are shown in **Table 1**. β-*actin* was employed as an internal reference. The *IL-16*, *IL-1*, *IgM*, *MHC-Iα*, and *TNF-α* gene expressions were normalized to β-*actin* as relative expression values using the $2^{-\Delta\Delta Ct}$ method (Pang et al., 2022).

Statistical Analysis

SPSS software version 21.0 was used for statistical analysis (SPSS Incorporation, United States). The data were analyzed using one-way ANOVA. Duncan's test was used to test mean comparisons. For identifying significant differences between groups, data are reported as mean ± SD and analyzed by the Student's *t*-test. When $p < 0.05$, the differences were judged significant.

RESULTS

Congo Red Assay

The recombinant vector pBAD-*VAGM001033* was successfully constructed and confirmed by PCR identification and DNA sequencing as shown in **Figure 1**. The *VAGM001033* gene consists of an ORF of 1,035 bp, encoding 344 amino acids with a GGDEF domain from amino acid 184–344. With the increase of the concentration of the L-arabinose, the red-stained, dry, and rough colonies were observed (**Figures 2A–C**), indicating that VAGM1033 synthesizes c-di-GMP as a diguanylate cyclase.

Identification and Characteristics of the Δ*VAGM001033* Mutant

The *VAGM001033* gene (locus in Chr1: 1104559-1105593) consists of an ORF of 1,035 bp, encoding 344 amino acids with a predicted GGDEF domain from amino acid 184-344. The Δ*VAGM001033* mutant was constructed by deleting the total ORF of the Δ*VAGM001033* gene based on genetic tools. The result of the successfully constructed Δ*VAGM001033* mutant was achieved without altering the remaining sequences by a PCR identification (**Figure 3**) and direct DNA sequencing.

Morphology was observed by transmission electron microscope (TEM) and no discernible morphological difference was observed between the wild type and Δ*VAGM001033* mutant. Both the wild type and Δ*VAGM001033* mutant had a rod shape and a single polar flagellum (**Figure 4**). Δ*VAGM001033* mutant showed a similar growth curve with the wild-type strain when cultured in the TSB medium (**Figure 5**). Wild type and Δ*VAGM001033* mutant grew in the exponential growth phase for 0–6 h and reached the stationary phase at 15 h ($OD_{600} \approx 2.0$).

The swarming circle diameter of Δ*VAGM001033* mutant (39.33 ± 0.57 mm) was significantly bigger than that in the wild type (**Figure 6** and **Table 2**), indicating that the swarming

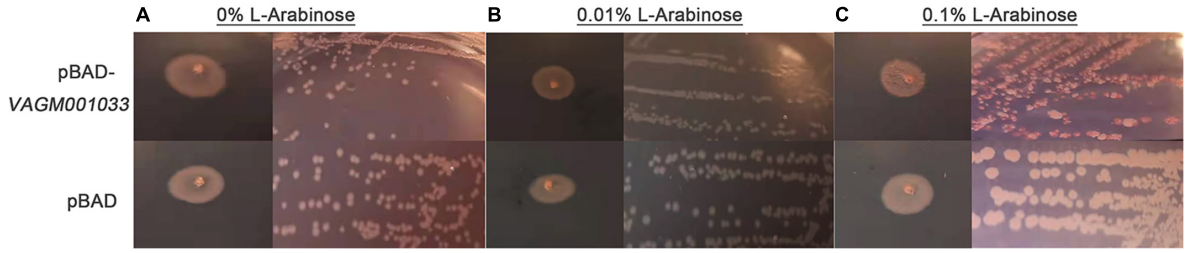

FIGURE 2 | Phenotypic characterization on Congo red plates. With the increase of the concentration of the inducing agent L-arabinose, the phenotype of the colony displays more obvious, the red-stained, dry, and rough form (**A:** 0% L-arabinose; **B:** 0.01% L-arabinose; and **C:** 0.1% L-arabinose).

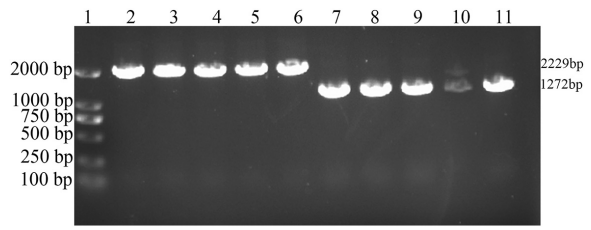

FIGURE 3 | PCR identification of Δ*VAGM001033* mutant. 1: DNA marker; 2–6: PCR amplifications with wild-type HY9901 genomic DNA (2,229 bp); 7–11: PCR amplifications with Δ*VAGM00133* mutant genomic DNA (1,272 bp).

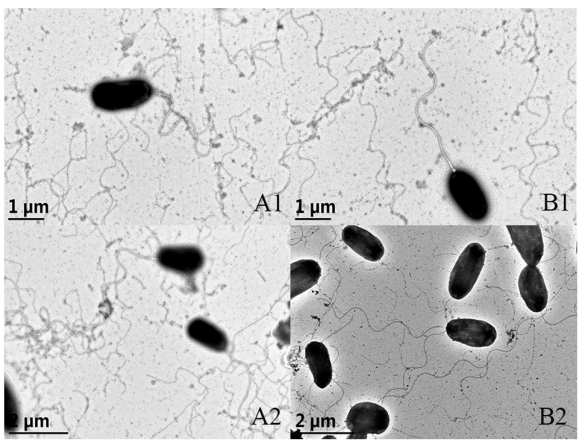

FIGURE 4 | Morphological characteristics of the wild-type strain (A1 × 25,000, A2 × 20,000) and the Δ*VAGM001033* mutant (B1 × 25,000, B2 × 20,000) *via* transmission electron microscope.

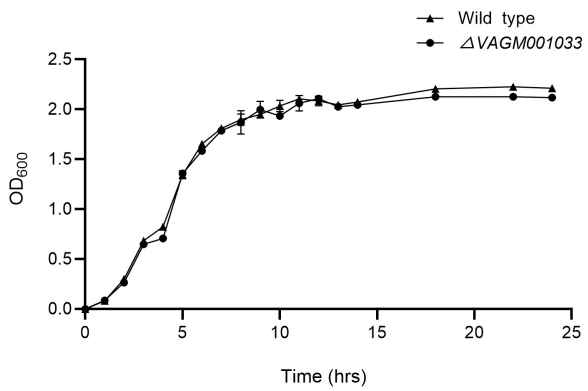

FIGURE 5 | The growth curve of the wild-type strain and the Δ*VAGM001033* mutant. At various time intervals, aliquots of cell culture were obtained and cell density was measured at OD_{600}.

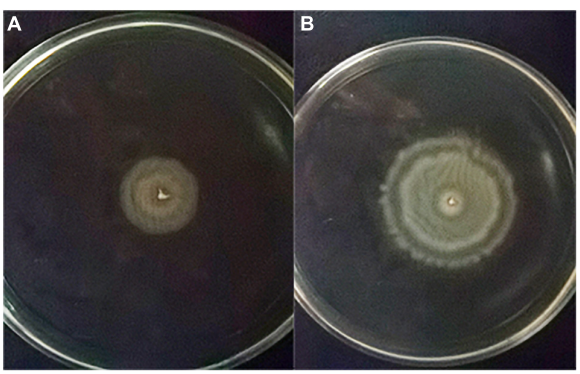

FIGURE 6 | Swarming circle diameter (mm) **(A)** the wild-type strain; **(B)** the Δ*VAGM001033* mutant.

ability of Δ*VAGM001033* mutant was significantly enhanced ($p < 0.01$). However, biofilm formation of *VAGM001033* mutant (0.29 ± 0.02) had significantly decreased when compared with that of the wild type ($p < 0.01$) (**Table 2**). The extracellular protease activity of Δ*VAGM001033* mutant (0.188 ± 0.02) was significantly lower than that of the wild type (0.308 ± 0.02) ($p < 0.05$) (**Table 2**). The LD_{50} value of Δ*VAGM001033* mutant was 100-fold higher than that of the wild type (**Table 2**), indicating that the virulence of Δ*VAGM001033* mutant was significantly decreased ($p < 0.01$). All the dead groupers revealed symptoms of vibriosis specifically characterized by hemorrhaging, skin lesions, and swelling in the liver and kidney. Re-isolated bacteria taken from the liver and spleen tissues were identified as *V. alginolyticus* by 16S rDNA. None of the fish died or were diseased in the control group.

TABLE 2 | Comparison of biological characteristics between HY9901 and the Δ*VAGM001033* mutant.

Characteristics	HY9901	Δ*VAGM001033*
Swarming motility (mm)	23.33 ± 0.57	39.33 ± 0.57**
Biofilm formation	0.73 ± 0.03	0.29 ± 0.02**
Activity of ECPase	0.308 ± 0.02	0.188 ± 0.02*
LD_{50} (CFU/ml)	1.42×10^5	3.47×10^{7}**

*Values are mean ± SD of triplicates. Significant differences between the ΔVAGM001033 mutant and V. alginolyticus HY9901 are indicated by asterisk *p < 0.05, **p < 0.01.*

Antibiotic Susceptibility

The results of the antimicrobial susceptibility are given in **Table 3**. Both the wild type and Δ*VAGM001033* mutant were sensitive to spectinomycin, ceftriaxone, chloramphenicol, SMZ/TMP, clarithromycin, tetracycline, norfloxacin, ofloxacin, and cefixime but resistant to polymyxin B, cefoxitin, cefuroxime, kanamycin, oxacillin, gentamicin, vancomycin, amikacin, and cefoperazone. In contrast, the mutant was sensitive to cefotaxime and nitrofurantoin, while the wild type was intermediate.

RNA-seq

To compare the transcriptomic profiles of the wild-type strain and Δ*VAGM001033* mutant, RNA-seq was conducted. A total of 756 differentially expressed genes (DEGs) were identified between these two strains. Of these DEGs (sequence accession number PRJNA794348), 109 were upregulated and 647 were downregulated in Δ*VAGM001033* mutant ($p < 0.05$, more than 2-fold enrichment, **Figure 7A**). Genes associated with flagellar assembly were identified as the most enriched pathway according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis (**Figure 7B**). Two-component system, ATP-binding cassette (ABC) transporters, and peptidoglycan biosynthesis were also significantly enriched (**Figure 7B**). Transcriptomic profiling analysis indicated that *VAGM001033* regulated multiple biological pathways, namely, flagellar motility, two-component system, ABC transporters, and peptidoglycan biosynthesis.

Analysis of Antibody Levels

Enzyme-linked immunosorbent assay experiment was performed to measure the specific antibody titer of *V. alginolyticus* in fish of Δ*VAGM001033* mutant and the PBS group. The result showed that specific antibodies in the Δ*VAGM001033* mutant group could be detected at the first week after the vaccination. The specific antibody titers of fish immunized with Δ*VAGM001033* mutant were significantly higher than those in the control group. During weeks 1 to 4 post-vaccination, $\log_2$ (antibody titers) in the sera of Δ*VAGM001033* mutant group all reached above 4.0 and the maximum reached 9.0, while that of the PBS group was always only 2.0 to 3.0. Comparison of the means of $\log_2$ (antibody titers) between Δ*VAGM001033* mutant and PBS control groups revealed a statistically significant difference ($p < 0.05$) (**Figure 8**).

TABLE 3 | Drug sensitivity test results of *V. alginolyticus* HY9901 and Δ*VAGM001033* mutant.

Antibiotic	Dose(μg)	Zone of inhibition (mm)			
		***V. alginolyticus* HY9901**	**Sensitivity**	**Δ*VAGM001033***	**Sensitivity**
polymyxin B	30	0	R	0	R
Cefoxitin	30	0	R	0	R
Cefuroxime	30	0	R	0	R
Kanamycin	30	0	R	0	R
Oxacillin	1	0	R	0	R
Gentamicin	10	0	R	0	R
Vancomycin	30	0	R	0	R
Cefuroxime	30	0	R	0	R
Amikacin	30	0	R	0	R
Cefoperazone	75	0	R	0	R
Spectinomycin	100	25 ± 0.2	S	35 ± 0.3	S
Ceftriaxone	30	23 ± 0.1	S	25 ± 0.2	S
Chloramphenicol	30	25 ± 0.2	S	33 ± 0.1	S
SMZ/TMP	23.75/1.25	20 ± 0.3	S	23 ± 0.2	S
Clarithromycin	15	20 ± 0.2	S	25 ± 0.3	S
Tetracycline	30	26 ± 0.2	S	28 ± 0.3	S
Norfloxacin	10	17 ± 0.2	S	20 ± 0.2	S
Ofloxacin	5	18 ± 0.3	S	20 ± 0.2	S
Cefixime	30	20 ± 0.2	S	23 ± 0.3	S
Cefotaxime	30	20 ± 0.2	I	23 ± 0.2	S
Nitrofurantoin	300	15 ± 0.1	I	20 ± 0.2	S

R, Resistance; I, Intermediate (Cefotaxime 15–22 mm; Nitrofurantoin 15–16 mm); S, Susceptible.

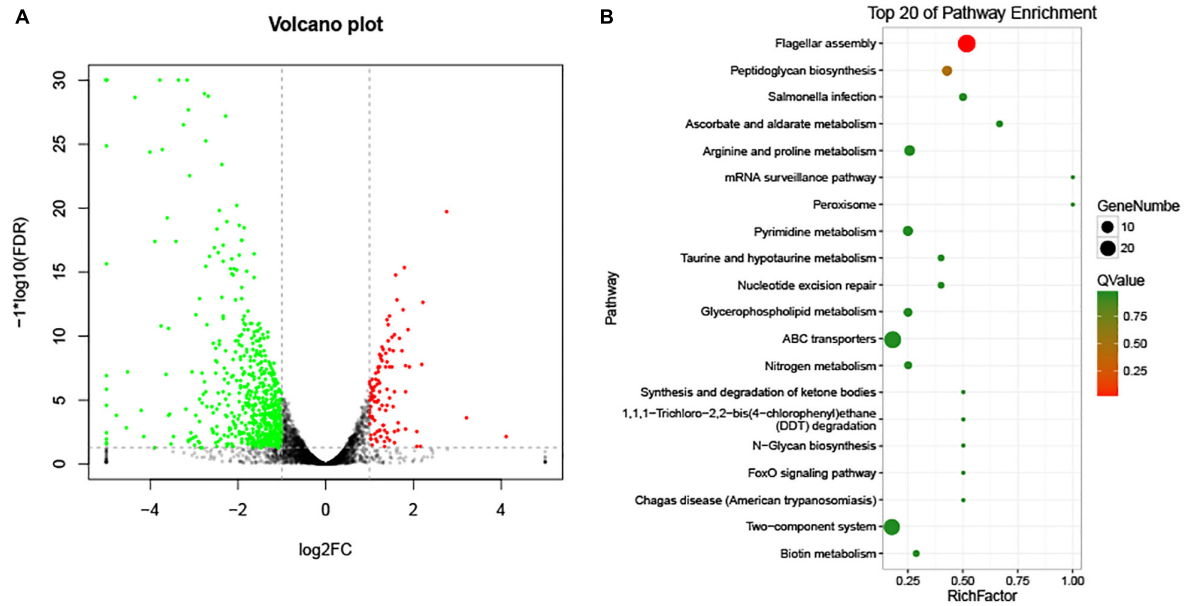

FIGURE 7 | RNA-seq results. **(A)** Volcano Plot; the significantly upregulated genes are represented by red dots, the significantly downregulated genes are represented by green dots, and the black and gray dots represent no significant differences. **(B)** KEGG pathway enrichment.

Histopathological Analysis

Groupers injected with *V. alginolyticus* exhibit sluggish behavior, inactive movements, curve or twirl their body. No clinical signs were observed in the Δ*VAGM001033* group and the PBS group. Histopathological analysis was conducted for the further observation that no histological abnormality was detected between the Δ*VAGM001033* mutant group and the PBS group. Nevertheless, distinct histological changes were observed in the *V. alginolyticus* HY9901 group (**Figure 9**). The intestine section of the *V. alginolyticus* HY9901 group revealed degeneration and necrosis of the mucosal epithelium, destruction of the intestinal microvilli, and cell debris in the lumen (**Figure 9A3**). The liver section of the *V. alginolyticus* HY9901 group showed vacuolation, hepatocyte disruption, and hepatic necrosis (**Figure 9B3**).

Immune Protective Effects of Δ*VAGM001033* in Pearl Gentian Grouper

Grouper were challenged with *V. alginolyticus* HY9901 at 42 days post-vaccination. The results revealed that the survival rate in the Δ*VAGM001033* mutant group was 84% with an RPS of 82% (**Figure 10**), which was significantly higher than as observed in the PBS group ($p < 0.01$).

Immune-Related Gene Expression Analysis

In this study, qPCR was conducted to evaluate the transcription levels of the immune-related genes. The immune-related genes of pearl gentian grouper, namely, *IgM*, *MHC-Iα*, *IL-1β*, *IL-16*, and *TNF-α* was significantly upregulated in the Δ*VAGM001033* group compared with those in the control group after vaccination (**Figure 11**).

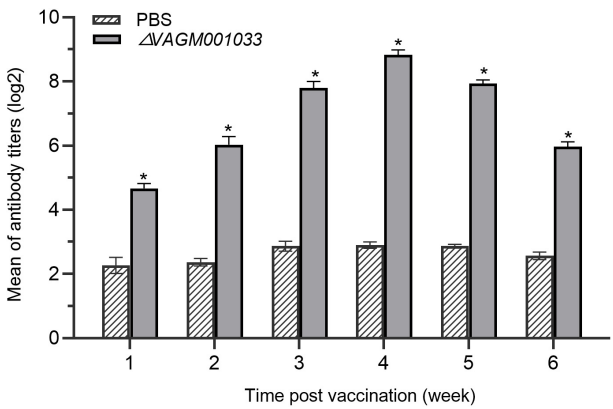

FIGURE 8 | ELISA evaluation of antibody levels in grouper vaccinated with PBS or Δ*VAGM001033* mutant. Sera samples were taken from week 1 to week 6 following immunization. The mean of $\log_2$ (antibody titers) was represented in each datum column, along with a standard error bar. The asterisks denoted significant changes between the vaccinated and control groups ($p < 0.05$).

DISCUSSION

Diguanylate cyclase is a class of essential enzymes responsible for the synthesis of cyclic di-GMP in bacteria. Numerous studies have linked GGDEF domain proteins to processes involved in motility, biofilm formation, and virulence (Ryjenkov et al., 2005). Despite many publications concerning GGDEF domains, detailed function analyses and potential mechanisms in *V. alginolyticus* are relatively scarce. The rising concentration of c-di-GMP caused an increased secretion of polysaccharides,

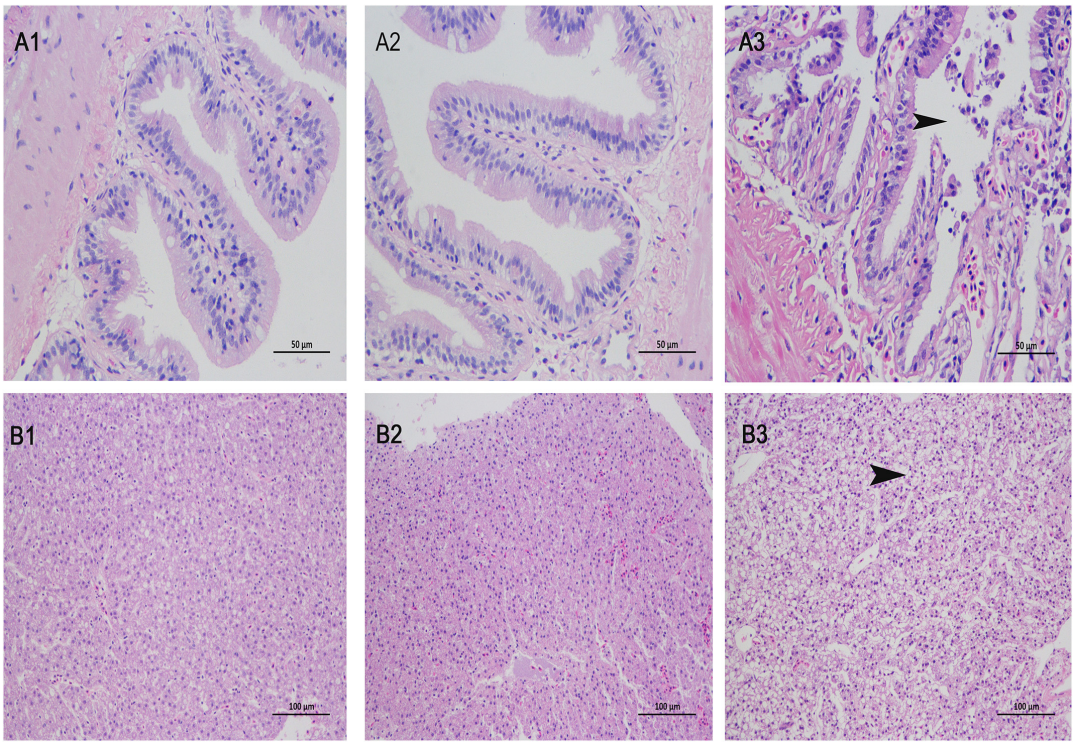

FIGURE 9 | Histological changes of intestine **(A1–3)** and liver **(B1–3)** stained with H&E. **(A1,B1)**, phosphate-buffered saline (PBS) group; **(A2,B2)**, Δ*VAGM001033* mutant group and **(A3,B3)**, *V. alginolyticus* HY9901 group. **(A1,2)**: intestine of the PBS group and the mutant group show intact intestinal mucosal epithelium, well-organized microvilli and no cell debris in the lumen (H&E staining; 400X). **(A3)**: intestine of the *V. alginolyticus* HY9901 group show hemorrhage, degeneration, and necrosis of the mucosal epithelium, destruction of the intestinal microvilli, and cell debris in the lumen (H & E staining; 400 ×). **(B1,2)**: liver of the PBS group and the mutant group show normal hepatocytes structure and organization (H&E staining; 200X). **(B3)**: liver of the *V. alginolyticus* HY9901 group show vacuolation, hepatocyte disruption, and hepatic necrosis (H&E staining; 200X).

which led to binding with Congo red to form a red-stained, dry, and rough colony morphology. Polysaccharides are important metabolites secreted by bacteria during their growth and metabolism (Kenne and Lindberg, 1983). In this study, L-arabinose was a chemical inducer that involved the regulation of the L-arabinose operon of *E. coli*. The arabinose system was often for practical use in protein expression systems because the *ara* promoter acted positively to stimulate transcription in the presence of arabinose (Schleif, 2010, 2000). It was observed that with the increase of the concentration of the L-arabinose, more secretion of polysaccharide is detected and the red-stained, dry, and rough form of the colony gets more obvious.

Motility was a necessity for bacteria to detect and pursue nutrients and to reach and maintain their appropriate niches for colonization (Wadhwa and Berg, 2021). In comparison with the wild-type strain, the mutant displayed enhanced swarming motility with a bigger swarm circle diameter. Biofilm is a structured consortium that causes chronic infections due to increased tolerance to antibiotics and disinfectant chemicals, and resisting phagocytosis and the host's immune defense system (Sharma et al., 2019; Vestby et al., 2020). Previous studies confirmed that HmsT in *Yersinia pesits* regulated biofilm formation by control of poly-β1,6-N-acetylglucosamine synthesis (Bobrov et al., 2011). Biofilm formation was significantly decreased in Δ*VAGM001033* mutant in this study.

Antimicrobial usage in aquaculture can result in residues of antimicrobials in seafood, which was a hazard to public health (Aly and Albutti, 2014). Δ*VAGM001033* mutant was sensitive to cefotaxime and nitrofurantoin, in contrast, the wild type was intermediate. Especially, the zone of inhibition of Δ*VAGM001033* mutant was bigger than that in the wild type, indicating that Δ*VAGM001033* mutant was more sensitive to the antibiotics. The results of antibiotic susceptibility experiments suggest the diguanylate cyclase VAGM001033 could regulate the antibiotic susceptibility through controlling the concentration of c-di-GMP and some receptors of c-di-GMP perform some important functions in the formation mechanism of drug resistance in *V. alginolyticus*.

A total of 756 DEGs were identified between the wild type and the mutant by RNA-seq. Flagellar assembly, two-component system, ABC transporters, and peptidoglycan biosynthesis were significantly enriched in the KEGG pathway, which was a database resource providing all knowledge about genomes and their relationships to biological systems such as cells and whole organisms and their interactions with the environment (Aoki-Kinoshita and Kanehisa, 2007). These results of RNA-seq suggest that VAGM001033 has

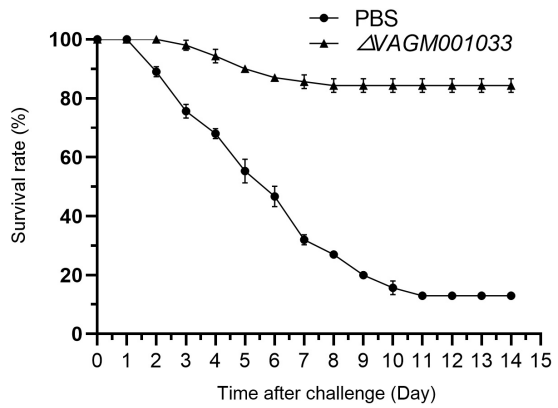

FIGURE 10 | Survival rate of grouper after challenge with *V. alginolyticus* HY9901. Control fish was injected with PBS (●). Fish was vaccinated with Δ*VAGM 001033* (▲).

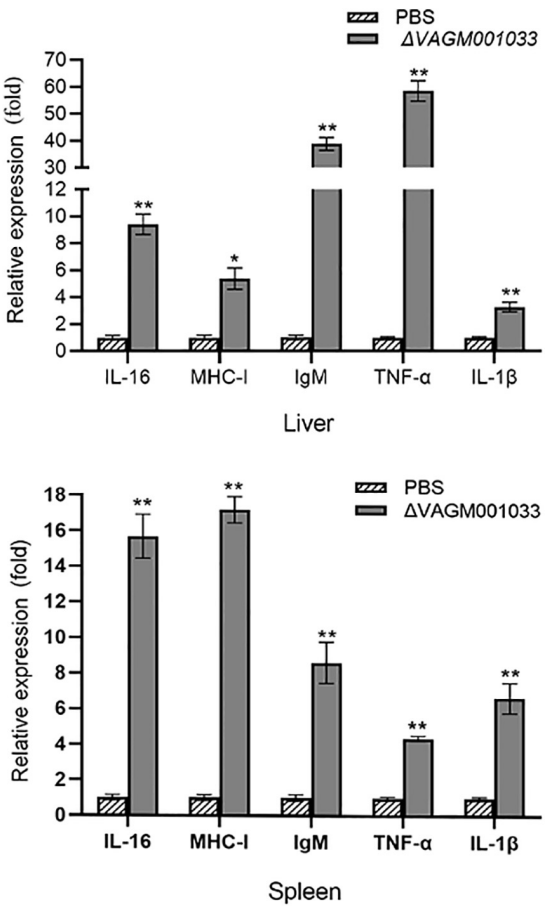

FIGURE 11 | Expression of immune-related genes in liver and spleen. The liver and spleen of pearl gentian grouper were sampled at 42 days post-immunization. Each immune-related gene's transcription level was compared to that of β-actin. The error bars showed the SD and the bars show the three biological replicates of mean relative expression. $^{*}p < 0.5$; $^{**}p < 0.01$.

controlled the biological phenotype such as swarming, biofilm forming, extracellular protease activity, virulence, and antibiotic susceptibility through regulating the above pathway. A previous study demonstrated that YedQ, a diguanylate cyclase, affected c-di-GMP-associated pathways, namely, flagellar assembly, siderophore pathway, exopolysaccharide biosynthesis pathway, and oxidative stress resistance in *Pseudomonas syringae via* RNA-sequencing (Wang et al., 2019). Taken together, the present data indicated that VAGM1033 might involve in regulating motility, biofilm formation, and virulence as diguanylate cyclase.

To evaluate the potential of the Δ*VAGM001033* mutant as a live attenuated vaccine candidate, a series of experiments were conducted. A previous study reported that the accessory colonization factor *acfA* deletion could induce a high antibody titer, upregulated the immune-relative genes, reached the RPS value of 81.1% without showing clinical symptoms and histopathological changes (Chen et al., 2019a). A study also confirmed a superoxide dismutase *sodB* deletion provided an effective immunoprotection with an RPS of 86.5% after vaccination, and also induced a high antibody titer (Chen et al., 2019b). More studies confirmed that live attenuated vaccines could provide significant immunoprotectivity against *Vibrio*, such as Δ*tyeA* mutant (Zhou et al., 2020), Δ*hop* mutant (Pang et al., 2018), Δ*clpP* mutant (Chen et al., 2020), and Δ*vscB* mutant (Pang et al., 2022). In this study, the live attenuated strain of *VAGM001033* deletion could induce a high titer of specific IgM and provide the RPS of 82% in pearl gentian grouper without any side effect, suggesting Δ*VAGM001033* mutant is an excellent candidate for the live attenuated vaccine.

The mRNA transcription levels of the immune-related genes, namely, *IgM*, *MHC-Iα*, *IL-1β*, *IL-6*, and *TNF-α* were conducted to analyze the underlying immune responses. IgM is the pioneering force against infection which is produced earliest during the adaptive immune response (Watts et al., 2001). Some previous studies reported that it was a major component of humoral immune response in teleosts and induced high expression in the spleen of vaccinated fish (Watts et al., 2001; Kim et al., 2007; Castro et al., 2013). Δ*VAGM001033* mutant significantly increased IgM expression levels in this study, explaining why higher serum IgM titers were recorded in the vaccinated fish. MHC-Iα is responsible for combining with the endogenous antigen peptide, and presenting antigen to $CD8^{+}$ T cells, which is distributed on all the surfaces of nucleated cells, platelets, and reticulocytes (Dijkstra et al., 2001; Šimková et al., 2006). Compared to the control group, the mRNA transcription level of MHC-Iα was significantly upregulated, indicating the recognition and presentation of *V. alginolyticus* could be involved in the MHC pathway. Cytokines are synthesized and secreted by immune cells and tissue cells, a class of soluble small molecules, namely, IL, IFN, TNF, CSF, GF, and chemokine. Cytokines can bind to the corresponding receptors to regulate the cellular activity, immune response, and inflammation (Secombes et al., 2001; Zou and Secombes, 2016; Sakai et al., 2021). In this study,

IL-1β, IL-6, and TNF-α were upregulated in different organs, indicating the activation of immune responses.

CONCLUSION

In summary, an in-frame deletion strain of Δ*VAGM001033* mutant was constructed successfully. The results showed that VAGM1033 was a key diguanylate cyclase in *V. alginolyticus*, which is involved in the regulation of motility, biofilm formation, and virulence. These results provided an essential reference for further study about the diguanylate cyclase in *V. alginolyticus*. Moreover, Δ*VAGM001033* mutant provided significant immunoprotection to grouper against *V. alginolyticus* by inducing a high antibody titer and upregulating immune-related genes without histopathological abnormality. The total data indicated that Δ*VAGM001033* mutant could be applied as a live attenuated vaccine to prevent and control fish diseases caused by *V. alginolyticus*.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the (https://www.ncbi.nlm.nih.gov/genbank/) repository, accession number PRJNA794348.

ETHICS STATEMENT

The animal study was reviewed and approved by Epidemiology for Aquatic Economic Animals Ethics Committee of Guangdong Ocean University.

AUTHOR CONTRIBUTIONS

HT conceived and designed the study, performed the experiment, generated the data and figures, and wrote the original draft. FD edited the data and reviewed the manuscript. GL and XW constructed the plasmids. JJ and SC made some critically contribution during the revision of the manuscript and also, they helped in the submission of sequences online. All authors contributed to the article and approved the submitted version.

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REFERENCES

Aly, S. M., and Albutti, A. (2014). Antimicrobials use in aquaculture and their public health impact. *J. Aquac. Res. Dev.* 5:247. doi: 10.4172/2155-9546.1000247

Amenyogbe, E., Huang, J., Chen, G., and Wang, W. (2021). Probiotic potential of indigenous (*Bacillus sp*. RCS1, Pantoea agglomerans RCS2, and Bacillus cereus strain RCS3) isolated from cobia fish (*Rachycentron canadum*) and their antagonistic effects on the growth of pathogenic *Vibrio alginolyticus*, *Vibrio harvey*. *Front. Mar. Sci.* 8:672213. doi: 10.3389/fmars.2021.672213

Aoki-Kinoshita, K. F., and Kanehisa, M. (2007). "Gene annotation and pathway mapping in KEGG," in *Comparative Genomics*, ed. N. H. Bergman (Totowa: Humana Press), 71–91. doi: 10.1007/978-1-59745-515-2_6

Bauer, A. W. (1966). Antibiotic susceptibility testing by a standardized single disc method. *Am. J. Clin. Pathol.* 45, 149–158. doi: 10.1093/ajcp/45.4_ts.493

Bobrov, A. G., Kirillina, O., Ryjenkov, D. A., Waters, C. M., Price, P. A., Fetherston, J. D., et al. (2011). Systematic analysis of cyclic di-GMP signalling enzymes and their role in biofilm formation and virulence in *Yersinia pestis*. *Mol. Microbiol.* 79, 533–551. doi: 10.1111/j.1365-2958.2010.07470.x

Cai, S., Cheng, H., Pang, H., Jian, J., and Wu, Z. (2018). AcfA is an essential regulator for pathogenesis of fish pathogen *Vibrio alginolyticus*. *Vet. Microbiol.* 213, 35–41. doi: 10.1016/j.vetmic.2017.11.016

Castro, R., Jouneau, L., Pham, H.-P., Bouchez, O., Giudicelli, V., Lefranc, M.-P., et al. (2013). Teleost fish mount complex clonal IgM and IgT responses in spleen upon systemic viral infection. *PLoS Pathog.* 9:e1003098. doi: 10.1371/journal.ppat.1003098

Chen, Y., Wu, F., Pang, H., Tang, J., Cai, S., and Jian, J. (2019b). 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

Chen, Y., Wu, F., Wang, Z., Tang, J., Cai, S., and Jian, J. (2019c). Construction and evaluation of *Vibrio alginolyticus* Δ*clpP* mutant, as a safe live attenuated vibriosis vaccine. *Fish Shellfish Immunol.* 98, 917–922.

Chen, Y., Cai, S., and Jian, J. (2019a). Protection against *Vibrio alginolyticus* in pearl gentian grouper (♀*Epinephelus fuscoguttatus* × ♂*Epinephelus lanceolatu*) immunized with an acfA-deletion live attenuated vaccine. *Fish Shellfish Immunol.* 86, 875–881. doi: 10.1016/j.fsi.2018.12.030

Chen, Y., Wu, F., Wang, Z., Tang, J., Cai, S., and Jian, J. (2020). Construction and evaluation of *Vibrio alginolyticus* Δ*clpP* mutant, as a safe live attenuated vibriosis vaccine. *Fish Shellfish Immunol.* 98, 917–922. doi: 10.1016/j.fsi.2019.11.054

Clinical and Laboratory Standards Institute [CLSI] (2013). *Performance standards for Antimicrobial Susceptibility Testing; Twenty-Third Informational Supplement (M100- S23-2013)*. Wayne: Clinical and Laboratory Standards Institute.

Cooper, M. D., and Alder, M. N. (2006). The evolution of adaptive immune systems. *Cell* 124, 815–822. doi: 10.1016/j.cell.2006.02.001

Dijkstra, J. M., Fischer, U., Sawamoto, Y., Ototake, M., and Nakanishi, T. (2001). Exogenous antigens and the stimulation of MHC class I restricted cell-mediated cytotoxicity: possible strategies for fish vaccines. *Fish Shellfish Immunol.* 11, 437–458. doi: 10.1006/fsim.2001.0351

Floyd, K. A., Lee, C. K., Xian, W., Nametalla, M., Valentine, A., Crair, B., et al. (2020). c-di-GMP modulates type IV MSHA pilus retraction and surface attachment in *Vibrio cholerae*. *Nat. Commun.* 11:1549. doi: 10.1038/s41467-020-15331-8

Gudding, R., and Van Muiswinkel, W. B. (2013). A history of fish vaccination: science-based disease prevention in aquaculture. *Fish Shellfish Immunol.* 35, 1683–1688. doi: 10.1016/j.fsi.2013.09.031

Hecht, G. B., and Newton, A. (1995). Identification of a novel response regulator required for the swarmer-to-stalked-cell transition in Caulobacter crescentus. *J. Bacteriol.* 177, 6223–6229. doi: 10.1128/jb.177.21.6223-6229.1995

Hueso-Gil, Á., Calles, B., and de Lorenzo, V. (2020). The Wsp intermembrane complex mediates metabolic control of the swim-attach decision of *Pseudomonas putida*. *Environ. Microbiol.* 22, 3535–3547. doi: 10.1111/1462-2920.15126

Jain, R., Sliusarenko, O., and Kazmierczak, B. I. (2017). Interaction of the cyclic-di-GMP binding protein FimX and the Type 4 pilus assembly ATPase promotes pilus assembly. *PLoS Pathog.* 13:e1006594. doi: 10.1371/journal.ppat.1006594

Joshi, A., Mahmoud, S. A., Kim, S.-K., Ogdahl, J. L., Lee, V. T., Chien, P., et al. (2020). c-di-GMP inhibits LonA-dependent proteolysis of TfoY in *Vibrio cholerae*. *PLoS Genet.* 16:e1008897. doi: 10.1371/journal.pgen.1008897

Kenne, L., and Lindberg, B. (1983). "Bacterial polysaccharides," in *The Polysaccharides*, ed. G. O. Aspinall (New York: Academic Press), 287–363. doi: 10.1016/B978-0-12-065602-8.50010-8

Kierek, K., and Watnick, P. I. (2003). Environmental determinants of *Vibrio cholerae* biofilm development. *Appl. Environ. Microbiol.* 69, 5079–5088. doi: 10.1128/AEM.69.9.5079-5088.2003

Kim, W. S., Nishizawa, T., and Yoshimizu, M. (2007). Non-specific adsorption of fish immunoglobulin M (IgM) to blocking reagents on ELISA plate wells. *Dis. Aquat. Organ.* 78, 55–59. doi: 10.3354/dao01843

Kimbrough, J. H., and McCarter, L. L. (2020). Identification of three new GGDEF and EAL domain-containing proteins participating in the Scr surface colonization regulatory network in *Vibrio parahaemolyticus*. *J. Bacteriol.* 203, e00409–20. doi: 10.1128/JB.00409-20

Lee, P.-T., Liao, Z.-H., Huang, H.-T., Chuang, C.-Y., and Nan, F.-H. (2020). β-glucan alleviates the immunosuppressive effects of oxytetracycline on the non-specific immune responses and resistance against *Vibrio alginolyticus* infection in *Epinephelus fuscoguttatus*× *Epinephelus lanceolatus* hybrids. *Fish Shellfish Immunol.* 100, 467–475. doi: 10.1016/j.fsi.2020.03.046

Luo, P., He, X., Liu, Q., and Hu, C. (2015). Developing universal genetic tools for rapid and efficient deletion mutation in vibrio species based on suicide t-vectors carrying a novel counterselectable marker, vmi480. *PLoS One* 10:e0144465. doi: 10.1371/journal.pone.0144465

Mathew, J. A., Tan, Y. P., Srinivasa Rao, P. S., Lim, T. M., and Leung, K. Y. (2001). *Edwardsiella tarda* mutants defective in siderophore production, motility, serum resistance and catalase activity. *Microbiology* 147, 449–457. doi: 10.1099/00221287-147-2-449

Pang, H., Chang, Y., Zheng, H., Tan, H., Zhou, S., Zeng, F., et al. (2022). A live attenuated strain of HY9901Δ*vscB* provides protection against *Vibrio alginolyticus* in pearl gentian grouper (♀*Epinephelus fuscoguttatus* × ♂*Epinephelus lanceolatu*). *Aquaculture* 546:737353. doi: 10.1016/j.aquaculture.2021.737353

Pang, H., Qiu, M., Zhao, J., Hoare, R., Monaghan, S. J., Song, D., 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

Ryjenkov, D. A., Tarutina, M., Moskvin, O. V., and Gomelsky, M. (2005). Cyclic diguanylate is a ubiquitous signaling molecule in bacteria: insights into biochemistry of the GGDEF protein domain. *J. Bacteriol.* 187, 1792–1798. doi: 10.1128/JB.187.5.1792-1798.2005

Sakai, M., Hikima, J., and Kono, T. (2021). Fish cytokines: current research and applications. *Fish. Sci.* 87, 1–9. doi: 10.1007/s12562-020-01476-4

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

Schleif, R. (2010). AraC protein, regulation of the l-arabinose operon in *Escherichia coli,* and the light switch mechanism of AraC action. *FEMS Microbiol. Rev.* 34, 779–796. doi: 10.1111/j.1574-6976.2010.00226.x

Secombes, C. J., Wang, T., Hong, S., Peddie, S., Crampe, M., Laing, K. J., et al. (2001). Cytokines and innate immunity of fish. *Dev. Comp. Immunol.* 25, 713–723. doi: 10.1016/s0145-305x(01)00032-5

Sharma, D., Misba, L., and Khan, A. U. (2019). Antibiotics versus biofilm: an emerging battleground in microbial communities. *Antimicrob. Resist. Infect. Control* 8:76. doi: 10.1186/s13756-019-0533-3

Šimková, A., Ottová, E., and Morand, S. (2006). MHC variability, life-traits and parasite diversity of European cyprinid fish. *Evol. Ecol.* 20, 465–477.

Su, Y.-L., Jiang, Y.-F., Chen, H.-J., Ye, S., Zhou, W.-H., Liu, H.-P., et al. (2021). Molecular characterization, expression and function analysis of *Epinephelus coioides* MKK4 response to SGIV and *Vibrio alginolyticus* infection. *Dev. Comp. Immunol.* 119:104020. doi: 10.1016/j.dci.2021.104020

Valentini, M., and Filloux, A. (2019). Multiple roles of c-di-GMP signaling in bacterial pathogenesis. *Annu. Rev. Microbiol.* 73, 387–406. doi: 10.1146/annurev-micro-020518-115555

Vestby, L. K., Grønseth, T., Simm, R., and Nesse, L. L. (2020). Bacterial biofilm and its role in the pathogenesis of disease. *Antibiotics* 9:59. doi: 10.3390/antibiotics9020059

Wadhwa, N., and Berg, H. C. (2021). Bacterial motility: machinery and mechanisms. *Nat. Rev. Microbiol.* doi: 10.1038/s41579-021-00626-4 [Epub ahead of print].

Wang, T., Cai, Z., Shao, X., Zhang, W., Xie, Y., Zhang, Y., et al. (2019). Pleiotropic effects of c-di-GMP content in *Pseudomonas syringae*. *Appl. Environ. Microbiol.* 85, e00152–19.

Wang, X., Du, Y., Yang, J., Tang, Y., and Luo, J. (2007). Preparation, characterization, and antimicrobial activity of quaternized chitosan / organic montmorillonite nanocomposites. *J. Biomed. Mater. Res. A* 84, 384–390. doi: 10.1002/jbm.a

Watts, M., Munday, B. L., and Burke, C. M. (2001). Immune responses of teleost fish. *Aust. Vet. J.* 79, 570–574. doi: 10.1111/j.1751-0813.2001.tb10753.x

Webster, S. S., Lee, C. K., Schmidt, W. C., Wong, G. C. L., and O'Toole, G. A. (2021). Interaction between the type 4 pili machinery and a diguanylate cyclase fine-tune c-di-GMP levels during early biofilm formation. *Proc. Natl. Acad. Sci. U. S. A.* 118:e2105566118. doi: 10.1073/pnas.2105566118

Wei, G., Tan, H., Ma, S., Sun, G., Zhang, Y., Wu, Y., et al. (2020). Protective effects of β-glucan as adjuvant combined inactivated *Vibrio harveyi* vaccine in pearl gentian grouper. *Fish Shellfish Immunol.* 106, 1025–1030. doi: 10.1016/j.fsi.2020.09.027

Windle, H. J., and Kelleher, D. (1997). Identification and characterization of a metalloprotease activity from *Helicobacter* pylori. *Infect. Immun.* 65, 3132–3137. doi: 10.1128/iai.65.8.3132-3137.1997

Wu, D. C., Zamorano-Sánchez, D., Pagliai, F. A., Park, J. H., Floyd, K. A., Lee, C. K., et al. (2020). Reciprocal c-di-GMP signaling: incomplete flagellum biogenesis triggers c-di-GMP signaling pathways that promote biofilm formation. *PLoS Genet.* 16:e1008703.

Xie, J., Bu, L., Jin, S., Wang, X., Zhao, Q., Zhou, S., et al. (2020). Outbreak of vibriosis caused by *Vibrio harveyi* and *Vibrio alginolyticus* in farmed seahorse *Hippocampus kuda* in China. *Aquaculture* 523:735168. doi: 10.1016/j.aquaculture.2020.735168

Zhou, S., Tu, X., Pang, H., Hoare, R., Monaghan, S. J., Luo, 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. Infect. Microbiol.* 10:183. doi: 10.3389/fcimb.2020.00183

Zou, J., and Secombes, C. J. (2016). The function of fish cytokines. *Biology (Basel)* 5:23. doi: 10.3390/biology5020023

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