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Cloing and Bioinformatics Analysis of *ndk* Gene from *Vibrio alginolyticus*

Yujia ZHANG[1,2△], Shi WANG[1,2△], Jian ZHONG[3], Weijie ZHANG[1,2], Xing XIAO[1,2], Zhiqing WEI[1,2], Huanying PANG[1,2*], Na WANG[4]

1. College of Fisheries, Guangdong Ocean University, Zhanjiang 524025, China; 2. Guangdong Provincial Key Laboratory of Aquatic Animal Disease Control and Healthy Culture / Key Laboratory of Control for Diseases of Aquatic Economic Animals of Guangdong Higher Education Institutes, Zhanjiang 524025, China; 3. Zhanjiang Customs, Zhanjiang 524022, China; 4. Chinese Academy of Inspection and Quarantine, Beijing 100176, China

Abstract [**Objectives**] The paper was to clone and analyze bioinformatics of *ndk* gene from *Vibrio alginolyticus*. [**Methods**] A pair of specific primers was designed based on the *ndk* gene sequence of *V. alginolyticus* HY9901. The full length of *ndk* gene was amplified by PCR and bioinformatics analysis was performed. MEGA 5.0 software was used to construct NDK phylogenetic tree by neighbor-joining method. SWISS-MODEL program was used to obtain the three-dimensional structural model of single subunit from NDK protein. [**Results**] The *ndk* gene, molecular structural formula $C_{702}H_{1094}N_{192}O_{214}S_7$, was 426 bp in total, encoding 141 amino acids, with the theoretical molecular weight of 15.871 99 kD and the theoretical *pI* value of 5.13. The prediction results of protein subcellular localization, SignalP 5.0, TMHMM Server 2.0 and SoftBerry-Psite showed that NDK mainly existed in the cytoplasm, and the protein was unstable and hydrophobic. There was neither signal peptide cleavage site, nor transmembrane region and KEGG metabolic pathway. The amino acid sequence had two protein kinase C phosphorylation sites, a casein kinase II phosphorylation site, a N-myristoylation site, three microbody C-terminal target signal sites, and a nucleoside diphosphate kinase active site. Homology analysis showed that the NDK of *V. alginolyticus* had high homology with that of *V. diabolicus*, with a similarity of 98.58%. Analysis of the structural functional domain revealed that the protein had one NDK structural functional domain. The prediction results of secondary structure showed that the α-helix, random coil, β-sheet and extended strand accounted for 53.19%, 28.37%, 7.09% and 11.35%, respectively. Analysis of NDK protein via STRING database demonstrated that the proteins interacting with NDK protein were NrdA, NrdB, GmK, CmK, TmK, PyrG, PyrH, RelA, FolE and SpoT. [**Conclusions**] The study plays a positive role in the prevention and control of vibriosis and the improvement of the current aquaculture environment.

Key words *Vibrio alginolyticus*, Gene cloning, *ndk*, Bioinformatics analysis

1 Introduction

In recent years, frequent outbreaks of vibriosis have seriously affected the development of aquaculture industry in China[1]. As one of the most common bacterial diseases[2], vibrio is widely distributed in the ocean, estuary and other water environment[3], and has a great impact on marine organisms. *Vibrio alginolyticus* is a kind of halophilic, thermophilic, facultative anaerobic gram-negative brevibacterium, without capsule or spore, which is one of the main dominant species in the marine environment[4]. When the water temperature is 25 - 35 ℃, *V. alginolyticus* easily causes serious vibriosis of fish, shrimp, shellfish and other aquatic economic animals[5-6]. This bacterium is also a zoonotic pathogen, and human ingestion of vibrio-contaminated food can cause diarrhea, otitis media, enteritis and other diseases[7-8]. The pathogenicity of *V. alginolyticus* depends on the interaction with the host, and its invasion and proliferation of the host cause tissue damage and disturb the metabolism of the host cells[9]. The main virulence factors of *V. alginolyticus* include extracellular products[10], lipopolysaccharides[11], iron carriers[12], and attachment factors. In addition, the pathogenicity of *V. alginolyticus* is also regulated by the type III secretion system[13], which is a hot spot of pathogenic bacteria research in recent years.

The type III secretion system (T3SS) is needle-like, and is a strictly controlled virulence mechanism. When *V. alginolyticus* infects the host, T3SS can directly inject virulence protein into the host cells, and interfere with the normal metabolism of the host cells, resulting in the death of the host cells[14]. Nucleoside diphosphate kinase (NDK), as an important enzyme of nucleoside triphosphate (NTPs) or its deoxidized derivatives, plays an important role not only in nucleotide metabolism, but also in bacterial growth, regulation of bacterial virulence and regulation of gene transcription[15]. Studies have shown that NDK is involved in the regulation of T3SS. ExsA is an important transcriptional regulatory protein of *Pseudomonas aeruginosa*, and plays an important leading role in regulating the activation of T3SS transcription; knocking out the *ndk* gene of *P. aeruginosa* can up-regulate *exsA*,

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△These authors contributed equally to this work.
Shi WANG, master candidate, research fields: aquatic animal medicine.
* Corresponding author. Huanying PANG, PhD., associate professor, research fields: aquatic veterinary medicine.

thereby regulating the transcription of T3SS genes[16]. Additionally, *ndk* is involved in adhesion to host cells. After the deletion of *ndk* gene, the biofilm formation ability of *Aeromonas veronii* and its invasion and adhesion ability in EPC cells were significantly reduced[17]. Previous studies indicate that *ndk* may regulate the expression of virulence related genes of *P. aeruginosa* at the transcriptional level. Transcriptomic sequencing showed that the deletion of *ndk* gene significantly inhibited the expression of *lasA*, *lasB*, *aprA*, *aprDEF*, *plcB* and *rhlABC* genes of *P. aeruginosa*[16]. In addition to playing an important regulatory role in bacterial virulence, *ndk* is also critical in regulating bacterial adaptability in the host. Studies have revealed that the NDK of *Mycobacterium tuberculosis* disrupts macrophage phagocytosis by hindering EEA1 recruitment to phagosomes and preventing the formation of mature phagosomes, or by inhibiting RILP recruitment to late phagosomes and preventing the formation of phagolysosomes[18]. Chopra *et al.* [19] have shown that NDK is an important cytotoxic factor secreted by *M. tuberculosis*, and can induce ATP-dependent P2Z receptor to mediate macrophage death.

In recent years, there have been many researches on NDK of *P. aeruginosa*, *M. tuberculosis* and *A. veronii* at home and abroad, but related researches of *ndk* in *V. alginolyticus* have not been reported yet. Therefore, in order to explore the function of *ndk* gene, the *ndk* gene of *V. alginolyticus* was cloned in this study, and its sequence was analyzed bioinformatically, which would lay a foundation for further research on the regulation mechanism of protein virulence to *V. alginolyticus*.

2 Materials and methods

2.1 Materials

2.1.1 Strains and vectors. *V. alginolyticus* HY9901 was a virulent strain preserved in Guangdong Provincial Key Laboratory of Aquatic Animal Disease Control and Healthy Culture, and *Escherichia coli* DH5α competence was also preserved by the laboratory. The clone vector pMD18-T was purchased from Takara.

2.1.2 Main reagents. Ex *Taq* DNA polymerase was derived from Takara. Bacterial genome DNA extraction kit and DNA glue recovery kit were purchased from Tiangen Biotech Co., Ltd., and other reagents were imported or domestic analytical pure. PCR primer synthesis and sequencing were completed by Sangon Biotech (Shanghai) Co., Ltd. The antibiotic ampicillin (Amp^+) was used at a concentration of 100 μg/mL.

2.2 Methods

2.2.1 Extraction of total DNA from *V. alginolyticus* HY9901. *V. alginolysticus* was spread on TSA plate. Single colonies were picked and inoculated on TSB medium, and oscillated at 28 ℃ for more than 12 h. Appropriately 1 mL of bacterial solution was loaded into a centrifuge tube and centrifuged at 5 000 rpm for 3 min to collect thalli. Genomic DNA was extracted according to the instructions of the kit, and stored at −20 ℃ for later use.

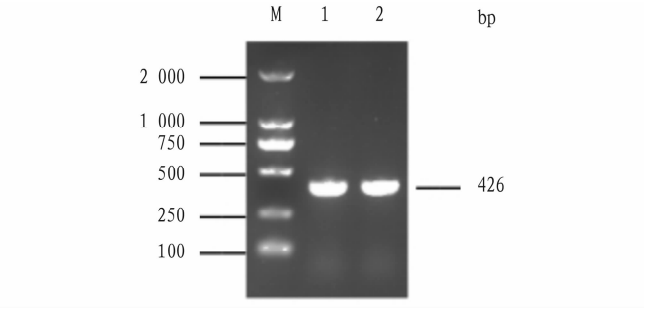
2.2.2 Cloning of *ndk* gene. According to the *ndk* gene sequence of *V. alginolyticus* (accession No.: CP098034) on Genbank, it was aligned with the full genome sequence of *V. alginolyticus* HY9901. A pair of primers was designed according to the *ndk* gene sequence of *V. alginolyticus* in Genbank. Forward primer P1: 5'-ATGGCTCTAGAAAGAACATTTTCA-3'; reverse primer P2: 5'-TTAGCGAGAGAAAACTTCTGATTCA-3'. Using the total DNA extracted from *V. alginolyticus* as a template, PCR was performed in the following procedures: pre-denaturating at 95 ℃ for 3 min; denaturating at 95 ℃ for 30 s, annealing at 59 ℃ for 30 s, extension at 72 ℃ for 40 s, 33 cycles; extension at 72 ℃ for 5 min. PCR products were detected by electrophoresis on 1% agarose gel and purified by DNA gel cutting kit.

2.2.3 Sequencing of PCR products. According to the instructions, PCR products were connected to pMD18-T vector, then transformed into *E. coli* DH5α competent cells, and screened on the Amp + resistant LB plate. Finally, positive clones were sent to Sango Biotech for sequencing.

2.2.4 Bioinformatics analysis of *ndk* gene from *V. alginolyticus* HY9901. Sequence homology alignment and similarity analysis were performed using NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi); nucleic acid homology was aligned via DNAMAN Version 6.0 (Lynnon Biosoft); ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and ExPASy Proteomics Server (http://ca.expasy.org) was used to derive amino acid sequence, determine open reading frame (ORF), calculate molecular weight (Mw), and predict theoretical isoelectric point (pI), *etc.*; signal peptide sequence was predicted by online analysis software SignalP 5.0 Server (http://www.cbs.dtu.dk/services/SignalP); transmembrane domain structure was predicted using TMHMM Server 2.0 (http://www.cbs.dtu.dk/services/TMHMM); the distribution of functional sites in amino acid sequences was predicted using SoftBerry-Psite (http://linux1.softberry.com/berry.phtml?topic=psite&group=programs&subgroup=proloc); the functional domain of protein structure was analyzed via SMART website (http://smart.embl-heidelberg.de/); subcellular localization was predicted using PSORT II Prediction (http://psort.hgc.jp/form2.html); Clastal 2.0 and MEGA 5.0 software was used to construct the phylogenetic tree by neighbor-joining method; SWISS-MODEL program of ExPASy server (http://www.swissmodel.expasy.org/) was used for modeling; KEGG website (https://www.genome.jp/kegg/) was used to search for metabolic pathways; STRING database was used to search protein network interaction (http://string.embl.de/).

3 Results and analysis

3.1 Full-length gene amplification The *ndk* gene was amplified by PCR assay. The amplified products were analyzed by agarose gel electrophoresis, and specific bands of about 426 bp were amplified (Fig. 1). Sequencing of the amplified product and the cloned vector pMD18-T showed that the *ndk* gene contained an open reading frame of 426 bp, encoding 141 amino acids. The amplified product of the gene was submitted to GenBank (accession No.: QQ620230).



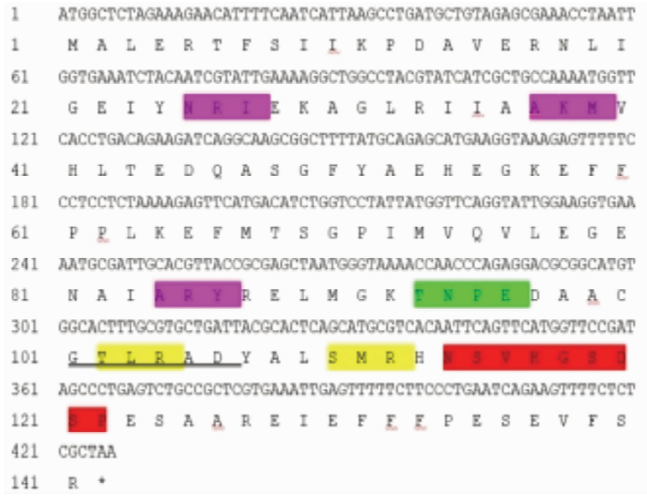
Note: M. DL 2000 DNA Marker; 1 – 2. PCR products of *ndk*.

Fig. 1 Amplification of *ndk* gene

3.2 Physicochemical properties The physicochemical properties of NDK protein of *V. alginolyticus* was analyzed by ExPASy software. The results showed that the total number of atoms was 2 209 and the molecular structure formula was $C_{702}H_{1094}N_{192}O_{214}S_7$. The theoretical molecular weight was 15.871 99 kD and the theoretical *pI* was 5.13. The instability coefficient was 48.94 (threshold value 40), so the protein was unstable. The fat coefficient was 775.53, the total mean hydrophilicity was −0.335, and the total hydrophobicity of the protein was low. The protein was free of tryptophan (Trp), pyrrolysine (Pyl), and selenocysteine (Sec). The total number of acid amino acid residues (Asp + Glu) was 23, and that of basic amino acid residues (Arg + Lys) was 16, and Met was at the N terminus. The half-life *in vivo* was longer than 20 h in yeast and longer than 10 h in *E. coli*, and the half-life *in vitro* was 30 h in mammalian reticular cells.

3.3 Sequence analysis The N-terminal signal peptide structure of the amino acid sequence of *ndk* gene was predicted by SignalP 5.0 Server program, and no signal peptide cleavage site was found in the gene. Prediction made by TMHMM Server 2.0 program showed that the protein had no transmembrane region. Prediction by SoftBerry-Psite demonstrated that the amino acid sequence contained two protein kinase C phosphorylation sites (102-104 aa, 110-112 aa), a casein kinase II phosphorylation site (93-96 aa), a N-myristoylation site (101-106 aa), three microbody C-terminal target signal sites (25-27 aa, 37-39 aa, 84-86 aa), and a nucleoside diphosphate kinase active site (114-122 aa) (Fig. 2). The prediction results of protein subcellular localization suggested that the NDK protein was most likely to be located in the cytoplasm (78.3%), followed by the nucleus (13.0%), and the probability of being located in mitochondria and peroxisome was 4.3%.

3.4 Homology and evolutionary analysis Homology analysis was performed using DNAMAN software. The NDK amino acid sequences of *V. anguillarum*, *V. campbellii*, *V. cholerae*, *V. diabolicus*, *V. fluvialis*, *V. harveyi*, *V. mimicus*, *V. parahaemolyticus* and *V. vulnificus* were aligned with that of *V. alginolyticus*. The results showed that the NDK of *V. alginolyticus* had high homology with those of other *Vibrio* strains, and the similarity with *V. diabolicus* was as high as 98.58% (Fig. 3). Multiple sequence similarity alignment indicated that the *ndk* gene in *Vibrio* strains was highly conserved.



Note: : Protein kinase C phosphorylation site; : Casein kinase II phosphorylation site; : N-myristoylation site; : Microbody C-terminal target signal site; : Nucleoside diphosphate kinase active site.

Fig. 2 Nucleotides of *ndk* gene and its encoded amino acid sequences

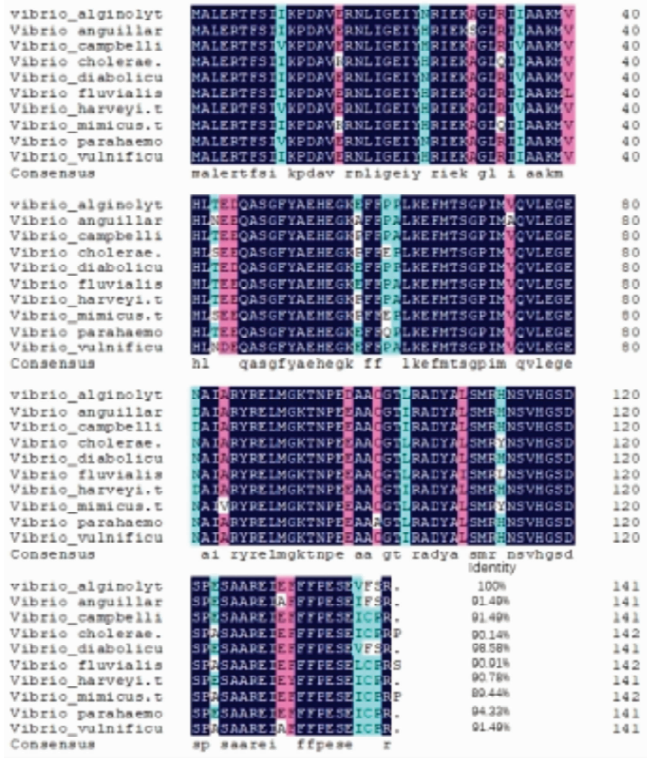


Fig. 3 Homology comparison of amino acid sequences of NDK protein from *Vibrio alginolyticus* and other *Vibrio* strains

The phylogenetic tree was constructed by combining the NDK amino acid sequence of *V. alginolyticus* and other *Vibrio* strains using the neighbor-joining method of MEGA 5.0. The results showed that *V. alginolyticus* and *V. diabolicus* clustered into the same subfamily, indicating that they were closely related, which was also consistent with the results of morphological and biochemical characteristics classification (Fig. 4).

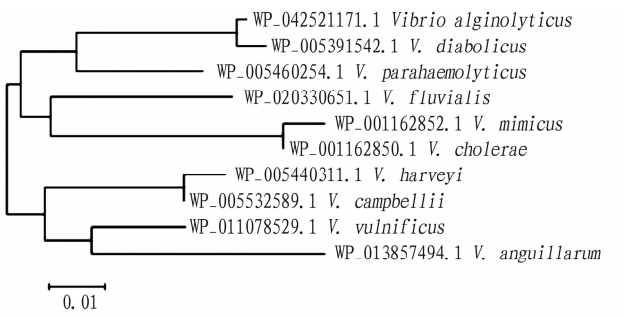


Fig. 4 Phylogenetic tree of NDK protein constructed by neighbor-joining method

3.5 Subunit structure The amino acid sequence of *ndk* gene was submitted to SWISS-MODEL program, and homologous proteins were automatically searched as templates to obtain the tertiary structure model of single subunit of NDK protein (Fig. 5).

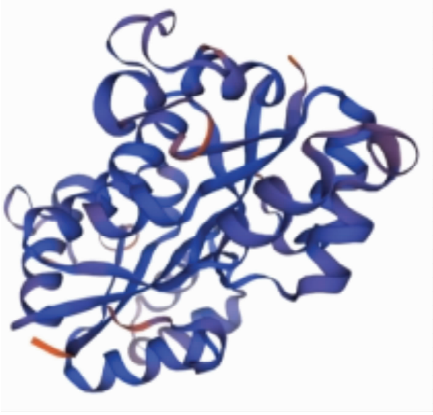


Fig. 5 Three-dimensional structural model of NDK protein subunit of *Vibrio alginolyticus*

3.6 Prediction of functional domain and secondary structure The functional domains of amino acid structure were analyzed using the SMART website program, and the prediction results showed that NDK protein had a functional domain, namely NDK (Fig. 6), which started at 3 aa and terminated at 140 aa, without hidden domains or functions.

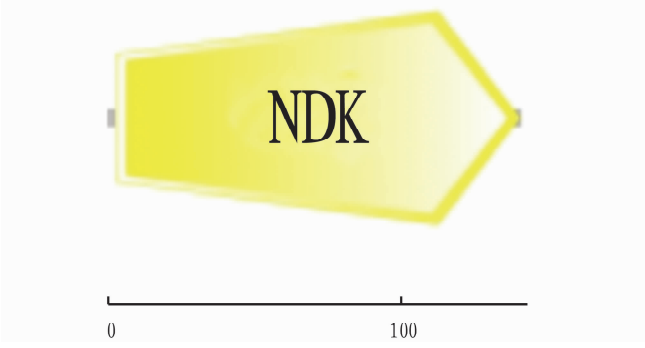
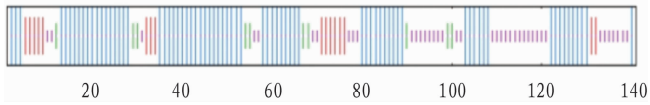


Fig. 6 Functional domain of NDK protein

The secondary structure of NDK protein was analyzed by SOPMA software. The results showed that the α-helix, random coil, β-sheet and extended strand accounted for 53.19%, 28.37%, 7.09% and 11.35%, respectively (Fig. 7).



Note: The line in blue represents α-helix; the line in purple represents random coil; the line in red represents extended strand; and the line in green represents β-sheet.

Fig. 7 Secondary structure of NDK protein

3.7 Network interaction of NDK protein The cross-linking analysis of NDK protein was performed using STRING database, and the results displayed that the main proteins interacting with NDK protein were NrdA, NrdB, GmK, CmK, TmK, PyrG, PyrH, RelA, FolE, and SpoT (Fig. 8).

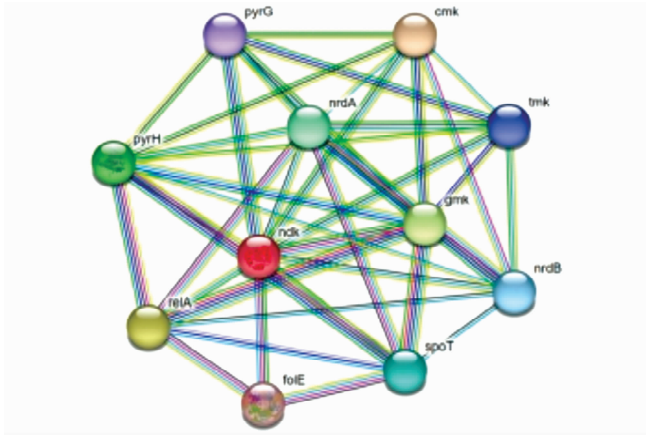


Fig. 8 Network interaction of NDK protein

4 Discussion

At present, bioinformatics analysis is an important method to analyze the structure and function of proteins, and can accurately predict the evolutionary relationship, physical and chemical properties and advanced structure of proteins[20-21]. Wang Sen[22] analyzed the NDK of *Aspergillus flavus* bioinformatically, and the results showed that there was no signal peptide or transmembrane region in NDK protein, which was similar to that in this study. In this study, the *ndk* gene of *V. alginolyticus* was cloned successfully and bioinformatic analysis was performed. The *ndk* gene was 426 bp in total, encoding 141 amino acids, with the theoretical molecular weight of 15.871 99 kD and the theoretical pI value of 5.13. This protein was unstable and hydrophobic. Through online prediction, it was found that there was no signal peptide, transmembrane region or KEGG metabolic pathway in *ndk* gene. The secondary structure was mainly composed of α-helix and a small amount of random coil, extended strand and β-sheet. Homology analysis indicated that NDK proteins were highly similar in *vibrio*, and the affinity with *V. diabolicus* was the closest. The amino acid sequence had two protein kinase C phosphorylation sites, a casein kinase II phosphorylation site, a N-myristoylation site, three microbody C-terminal target signal sites, and a nucleoside diphosphate kinase active site. Phosphorylation of protein is a post-translational modification, and phosphorylation of histidine plays a key role in the regulation of cells in prokaryotes. The NDK that

produces NTP transfers phosphoric acid from the active site to the histidine residue of the substrate protein, thereby playing the role of histidine kinase[23]. *V. alginolyticus* lacks endoplasmic reticulum and Golgi apparatus, and can not amidate or glycosylate the translated protein, but this does not affect phosphorylation modification[24].

In recent years, the prevalence of vibriosis has brought huge economic losses to aquaculture industry. The use of antibiotics has caused increasing problems such as bacterial resistance and drug residues[25]. NDK is a multifunctional protein that plays a crucial role in nucleotide metabolism, regulation of bacterial virulence, transcription and expression of virulence genes, regulation of apoptosis, regulation of inflammation, *etc.*[23]. At present, the *ndk* gene of *V. alginolyticus* has not been studied, so the in-depth exploration of *ndk* gene will play a positive role in the prevention and control of vibriosis and the improvement of the current aquaculture environment.

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