Cloning and Bioinformatic Analysis of the *fbpA* Gene in *Vibrio alginolyticus*

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Abstract [**Objectives**] To clone the *fbpA* gene from *Vibrio alginolyticus* strain HY9901 and perform bioinformatic analyses, aiming to preliminarily elucidate the structure and function of the FbpA protein. [**Methods**] The *fbpA* gene was amplified using PCR and sequenced. Bioinformatics software was employed to analyze the *fbpA* gene sequence and the deduced FbpA protein for physicochemical properties, signal peptides, transmembrane structures, functional sites, subcellular localization, homology comparisons, phylogenetic analysis, and structural prediction. [**Results**] The *fbpA* gene (1 014 bp, encoding 337 amino acids, GenBank accession number PP707017) was successfully cloned. The FbpA protein was identified as a stable hydrophilic protein (molecular weight 37.589 kD, pI 5.97) containing a signal peptide, lacking transmembrane domains, and predicted to be localized extracellularly. It harbors 7 N-myristoylation sites, 8 phosphorylation sites, 2 N-glycosylation sites, and 7 microbody C-terminal targeting signal sites. The protein is highly conserved within the *Vibrio* genus, exhibiting 99.41% and 99.40% similarity to the *Vibrio diabolicus* subgroup and *Vibrio antiquarius*, respectively, with all three clustering together on the same evolutionary branch. Secondary structure prediction indicated a predominance of alpha-helices (49.85%) and random coils (30.27%). [**Conclusions**] This study successfully cloned the *fbpA* gene and characterized the structural features and evolutionary relationships of the FbpA protein, laying a foundation for further investigation into its role in the pathogenesis of *V. alginolyticus* and the development of vaccines.

Key words *Vibrio alginolyticus*, Gene amplification, *fbpA* gene, Bioinformatics analysis

0 Introduction

Vibrio alginolyticus, belonging to the genus *Vibrio* within the family Vibrionaceae, is a halophilic and thermophilic, facultatively anaerobic marine bacterium[1]. This bacterium is one of the primary opportunistic pathogens causing vibriosis in marine aquaculture animals in southern China[2], and is widely distributed in aquatic environments such as oceans, estuaries, and mariculture ponds. It thrives within a temperature range of 17 – 42 ℃, with an optimum of 25 – 32 ℃, and a pH range of 6 – 8.5; it also exhibits strong halophilicity, tolerating salinities from 5‰ to 50‰[3].

V. alginolyticus is a zoonotic pathogen affecting both humans and aquatic animals, and represents a significant pathogen in cultured aquatic products. Outbreaks of this bacterium can cause mass mortality in fish, shrimp, and shellfish, resulting in substantial economic losses to the aquaculture industry[4]. Current control measures for vibriosis in aquatic animals primarily include chemoprophylaxis (*e.g.*, antibiotics), immunoprophylaxis (*e.g.*, vaccines), and biological control (*e.g.*, probiotics). However, the long-term use of antibiotics leads to problems such as drug residues and increased bacterial resistance. Furthermore, the number of licensed vibrio vaccines for aquaculture is limited; currently, only five commercial vaccines are available internationally for preventing vibriosis in fish[5]. Studies have demonstrated that *V. alginolyticus* isolates from various sources commonly exhibit multidrug resistance, with particularly pronounced resistance to penicillin and ampicillin[6].

The FbpA protein encoded by the *fbpA* gene in *V. alginolyticus* HY9901 is a significant intracellular transport protein, playing a crucial role in bacterial iron metabolism and virulence[7]. The expression and regulation of this gene significantly impact the growth, metabolism, and pathogenicity of Vibrio species. Studies have reported that the presence of synergistic anions such as phosphate or sulfate facilitates FbpA-mediated iron sequestration[8]. In protein dynamics, the *fbpA* gene, a homolog of *Borrelia* lipoprotein, inhibits the initiator protease of the classical complement pathway and promotes the function of bacterial immune evasion proteins[9]. The *fbpA* gene is of paramount importance. In-depth investigation into its structure, function, and expression regulation will enhance our understanding of its functional roles and mechanisms within organisms, thereby providing a vital foundation and rationale for future biomedical research and applications.

Consequently, cloning and performing bioinformatic analysis of the *fbpA* gene in *V. alginolyticus* HY9901 will not only facilitate a deeper understanding of the pathogenic mechanisms of this bacterium but also provide valuable insights for developing novel antibacterial agents or therapeutic strategies. This holds significant theoretical and practical importance for maintaining the health of

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marine ecosystems, preventing and treating diseases caused by *V. alginolyticus*, and advancing marine biotechnology.

1 Materials and methods

1.1 Materials

1.1.1 Strains. The highly virulent strain *V. alginolyticus* HY9901 was isolated from diseased crimson snapper (*Lutjanus erythropterus*) in the Zhanjiang sea area of Guangdong Province by our laboratory and maintained in culture[10].

1.1.2 Main reagents. ExTaq DNA Polymerase was purchased from TaKaRa. Bacterial Genomic DNA Extraction Kits and DNA Gel Extraction Kits were purchased from Tiangen Biotech Co., Ltd. All other reagents were of analytical grade, sourced domestically or internationally.

PCR primer synthesis and DNA sequencing were performed by Sangon Biotech (Shanghai) Co., Ltd. The working concentration of the antibiotic ampicillin (Ap) was 100 μg/mL.

1.1.3 Instruments. PCR Thermal Cycler and Electrophoresis System (Bio-Rad Laboratories, USA); Refrigerated High-Speed Centrifuge (Eppendorf Life Science, Germany); Gel Imaging System (ProteinSimple, USA); UV-Vis Spectrophotometer (SHIMADZU, Japan); Ultra-Low Temperature Freezer (Thermo Fisher Scientific, USA); HVE-50 Series Autoclave (HIRAYAMA, Japan); Ultrapure Water System (Beijing Leader Water Treatment Equipment Co., China).

1.2 Methods

1.2.1 Extraction of genomic DNA from *V. alginolyticus* HY9901. *V. alginolyticus* was retrieved from the ultra-low temperature freezer and streaked onto Tryptic Soy Agar (TSA) plates. Single colonies were selected and inoculated into Tryptic Soy Broth (TSB) medium at a 1 : 100 dilution. Cultures were incubated at 28 ℃ with shaking at 180 rpm for over 14 hours. An aliquot of the bacterial culture was transferred into an EP tube. Cells were harvested by centrifugation at 10 000 rpm for 2 – 3 min using a high-speed centrifuge. The supernatant was discarded, and the bacterial pellet was collected. Total genomic DNA was extracted from *V. alginolyticus* following the instructions of the Bacterial Genomic DNA Extraction Kit and stored at – 20 ℃ for subsequent use.

1.2.2 Cloning of the *fbpA* gene. A pair of primers targeting the *fbpA* gene of *V. alginolyticus* was designed based on its sequence. The forward primer P1 was: TTAAAGGTCAAA. The reverse primer P2 was: AGATGAAGTGAA. PCR amplification was performed using the extracted total DNA from *V. alginolyticus* HY9901 as the template. The PCR reaction conditions were as follows: initial denaturation at 95 ℃ for 5 min; followed by 30 cycles of denaturation at 95 ℃ for 30 sec, annealing at 60 ℃ for 30 sec, and extension at 68 ℃ for 30 sec; and a final extension at 72 ℃ for 10 min. The PCR products were analyzed by electrophoresis on a 1% agarose gel. Target DNA bands were excised from the gel and purified using a Gel Extraction Kit. The purified fragments were cloned into the pMD18-T vector and subsequently sent for sequencing.

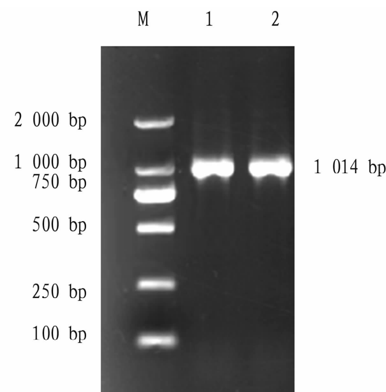
1.2.3 Bioinformatics analysis of *V. alginolyticus* HY9901 FbpA[11]. Sequence homology searches and similarity analyses were conducted using NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Amino acid sequence alignments were performed using DNAMAN Version 5.0 (Lynnon Biosoft). The ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and ExPASy Proteomics Server (http://ca.expasy.org) were used to deduce the amino acid sequence, identify the open reading frame (ORF), and predict the calculated molecular weight (Mw) and theoretical isoelectric point (pI). Signal peptide sequences were predicted using the online tool SignalP 5.0 Server (https://services.healthtech.dtu.dk/services/SignalP-5.0/#main-content).

Transmembrane domains were predicted using TMHMM Server 2.0 (https://dtu.biolib.com/DeepTMHMM). Functional site distributions within the amino acid sequence were predicted using SoftBerry-Psite (http://linux1.softberry.com/berry.phtml? topic=psite&group=programs&subgroup=proloc).

Protein structural and functional domains were analyzed using SMART (http://smart.embl-heidelberg.de/). Subcellular localization was predicted using PSORT II Prediction (http://psort.hgc.jp/form2.html). Phylogenetic trees were constructed using the neighbor-joining method within NCBI and MEGA 5.0 software. Protein modeling and analysis were performed using the SWISS-MODEL program[12] on the ExPASy server (http://www.swissmodel.expasy.org/).

2 Results and analysis

2.1 Full-length cloning of the *fbpA* gene The *fbpA* gene was amplified using PCR technology. Analysis of the amplification products by agarose gel electrophoresis revealed a specific band (Fig. 1). The amplified product was cloned into the pMD18-T vector and subjected to sequencing analysis. Results showed that the *fbpA* gene contains a 1 014 bp open reading frame (ORF), encoding 337 amino acids. The gene sequence was submitted to GenBank under the accession number PP707017.



NOTE M: DNA molecular weight marker (DL2000); 1: Amplified product; 2: Negative control.

Fig. 1 Amplification of the *Vibrio alginolyticus fbpA* gene

2.2 Physicochemical properties of FbpA Analysis of the *V. alginolyticus* FbpA protein using the ExPASy tool revealed a

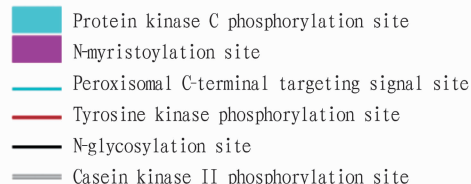
total atom count of 5 288 and a molecular formula of $C_{1\,672}H_{2\,645}N_{445}O_{514}S_{12}$. The theoretical molecular weight was calculated to be 37.589 kDa, with a theoretical isoelectric point (pI) of 5.97. The instability index was determined to be 28.48 (<40), indicating that it is a stable protein. The aliphatic index was 80.80, and the grand average of hydropathicity (GRAVY) was −0.448, suggesting that the protein exhibits hydrophilic properties. The protein does not contain pyrrolysine (Pyl). Its molar extinction coefficient at 280 nm is 42 985 $M^{-1} \cdot cm^{-1}$. Amino acid composition analysis showed a total of 48 acidic residues (Asp + Glu) and 45 basic residues (Arg + Lys). The protein sequence initiates with methionine (Met) at its N-terminus. Half-life predictions indicated that the protein retains stability for over 20 h in yeast, over 10 h in Escherichia coli, and exhibits a half-life of 30 h when expressed *in vitro* in mammalian reticulocytes.

2.3 Sequence analysis of FbpA Prediction of the N-terminal signal peptide structure using the SignalP 5.0 Server identified a clear signal peptide cleavage site, confirming the presence of a signal peptide. Analysis with the TMHMM Server 2.0 predicted that the protein lacks transmembrane domains. Prediction using the SoftBerry-Psite program revealed the presence of 7 N-myristoylation sites, 8 phosphorylation sites (comprising 5 protein kinase C phosphorylation sites, 4 casein kinase II phosphorylation sites, and 3 tyrosine kinase phosphorylation sites), 2 N-glycosylation sites, and 7 peroxisomal C-terminal targeting signal sites within the amino acid sequence (Fig. 2). Protein subcellular localization prediction indicated that FbpA is most likely localized extracellularly (including the cell wall), with a probability of 55.6%. The next most probable location is the cytoplasm (22.2%), while localization to the endoplasmic reticulum and vacuoles was each assigned a probability of 11.1%.

2.4 Homology and phylogenetic analysis of FbpA Homology alignment analysis of the *V. alginolyticus* FbpA protein amino acid sequence with other *Vibrio* strains was performed using DNAMAN software. Strains included in the alignment encompassed *Lutjanus erythropterus*, *Vibrio parahaemolyticus*, *Vibrio antiquarius*, the *Vibrio diabolicus* subgroup, *Vibrio alfacsensis*, *Vibrio owensii*, *Vibrio rotiferianus*, among others. The results demonstrated that the *V. alginolyticus* FbpA protein shares high sequence similarity (99.41%) with the FbpA protein from the *Vibrio diabolicus* subgroup. The next highest similarity was observed with *Vibrio antiquarius* at 99.40% (Fig. 3).

To further elucidate the evolutionary relationships of the *V. alginolyticus* FbpA protein with other Vibrio species, a phylogenetic tree was constructed using the Neighbor-Joining method in MEGA 5.0, based on the deduced FbpA amino acid sequences from *V. alginolyticus* and the other Vibrio strains. The results revealed that *V. alginolyticus* clusters within the same clade as the *Vibrio diabolicus* subgroup and *Vibrio antiquarius* (Fig. 4).

1 ATGAAAAAACTGCTAACTCTTTCAGCACTAACTTGCGCTACTCTAGCTCCAACAGCAATG
1 M K K L L T L S A L T C A T L A P T A M
61 GCTGCTGAAGAAGTGAACGTCTACTCTTACCGTCAACCTTTCCTAGTTGAACCTATGTTC
21 A A E E V N V Y S Y R Q P F L V E P M F
121 AATGAGTTCACTAAAGAAACTGGTATCAAAGTAAACGTAAAATTTGCTAAAAAAGGCTTA
41 N E F T K E T G I K V N V K F A K K G L
181 GCAGAAAAGCTAGCTCAAGAAGGTGAGTACAGCCCTGCTGACGTTATTCTTACGACAGAC
61 A E K L A Q E G E Y S P A D V I L T T D
241 ATCAGCCGTCTAGCTGAACTGACAAACAAAGGTTTAGTTCAGAAAGTCGATAGCAAAATC
81 I S R L A E L T N K G L V Q K V D S K I
301 ATTGAAGAAAACGTACCTGCTCAATACCAAGATAAAGAGAATGAGTGGTTTGCTCTAACT
101 I E E N V P A Q Y Q D K E N E W F A L T
361 CTACGTTCTCGTAGCGTTTATTCATCACGTGATCGCGTAGGTAAACTAGGCGCAGATTTC
121 L R S R S V Y S S R D R V G K L G A D F
421 AACTACGCAGACTTAGCTAAGCCAGAGTTCAAAGGTAAAATTTGTACTCGTAGCGGTAAG
141 N Y A D L A K P E F K G K I C T R S G K
481 CACCCGTACAACGTTTCTCTAGTTTCAGCAATGATTGCTCACAATGGCGAAGCAGAAACC
161 H P Y N V S L V S A M I A H N G E A E T
541 AAAGAGTGGTTAGAAGGCGTGAAAGCAAACCTTGCTCGCAAACCACAAGGCAACGATCGT
181 K E W L E G V K A N L A R K P Q G N D R
601 GCACAAGTTAAAGCGATCAAAGAAGGCCTATGTGATGTTTCTCTAGGTAACAGCTACTAC
201 A Q V K A I K E G L C D V S L G N S Y Y
661 CTAGGTAAAATGGTTAACGACAAAGAGCAAAAAGCTTGGGCTGATGCAGTTTACATCAAC
221 L G K M V N D K E Q K A W A D A V Y I N
721 TTCCCTAACCAAGAGACTACAGGCACTCACGTGAATATCTCTGGTATGGCGATGGCTAAG
241 F P N Q E T T G T H V N I S G M A M A K
781 TACTCTCCAAACAAAGACAATGCTGTGAAGCTAATGGAATTCCTAACGGGTGACAAAGCG
261 Y S P N K D N A V K L M E F L T G D K A
841 CAGCAAATGTACGCAGAAGTTAACTACGAGTACCCAGTTAAAGAAGGCGTGAAGCGTTCT
281 Q Q M Y A E V N Y E Y P V K E G V K R S
901 GAGCTTGTGGAATCTTGGGGTGACTTTAAAGCAGACAAGCTTTCTCTAGACGATATTGCA
301 E L V E S W G D F K A D K L S L D D I A
961 GCCAACCACGAAGCGGCAATTAAGCTATTAGATGAAGTGAAATTTGACCTTTAA
321 A N H E A A I K L L D E V K F D L *



NOTE The symbol * denotes the stop codon.

Fig. 2 Amino acid sequence of FbpA protein and predicted functional sites

2.5 Prediction of functional domains, secondary and tertiary structures of FbpA Prediction of functional domains within the amino acid sequence using the SMART website identified the presence of one Pfam domain (Fig. 5). Secondary structure prediction indicated the following composition: alpha helix (49.85%), random coil (30.27%), extended strand (14.24%), and beta turn (5.64%) (Fig. 6).

The FbpA amino acid sequence was submitted to the SWISS-MODEL program, which automatically searched for homologous protein templates, resulting in a predicted tertiary structure model of the FbpA monomer (Fig. 7).

2.6 FbpA protein interaction network Within the protein interaction network, proteins found adjacent to FbpA include FbpC, YpdA, BtuF, ANP65686.1, ANP66619.1, and ANP66618.1.

Co-occurrence interactions involved FbpC, ANP65686.1, and ANP65081.1. Co-expression interactions included FbpC, FtuF, HutZ, ANP66545.1, ANP66618.1, ANP65081.1, ANP66619.1, and ANP65686.1. Experimentally determined interactions included YpdA. Database-curated interactions involved FbpC, ANP65686.1, ANP66619.1, ANP66618.1, and ANP66617.1. Text mining-derived interactions included FbpC, BtuF, ANP65686.1, ANP66619.1, ANP66618.1, and ANP66617.1 (Fig. 8).

Fig. 3 Homology comparison of the deduced amino acid sequences of the *fbpA* gene

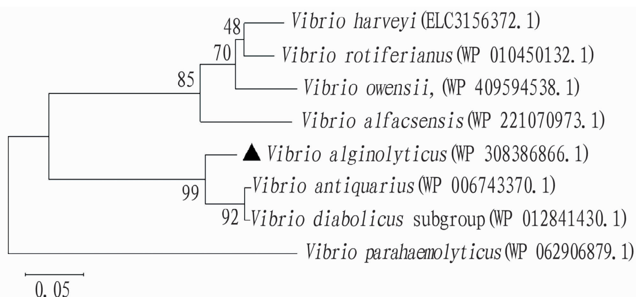


Fig. 4 Phylogenetic tree of FbpA amino acid sequences constructed using the NJ method

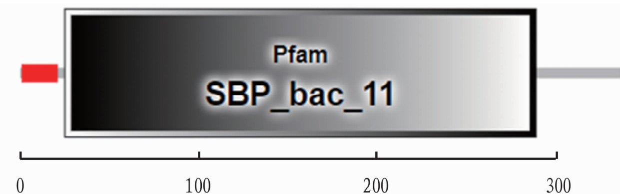
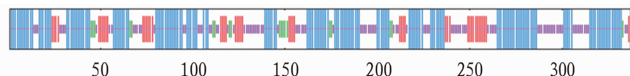


Fig. 5 Functional domains of FbpA



NOTE Purple: Random coil; Red: Extended strand; Blue: Alpha helix; Green: Beta turn.

Fig. 6 Secondary structure prediction of FbpA

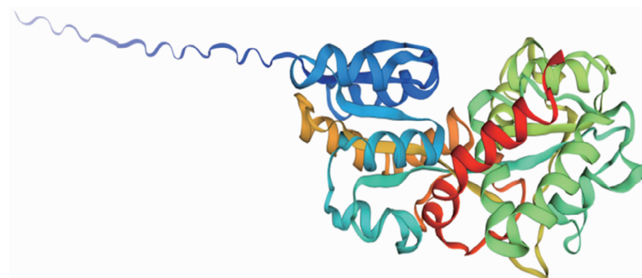


Fig. 7 Tertiary structure prediction of FbpA

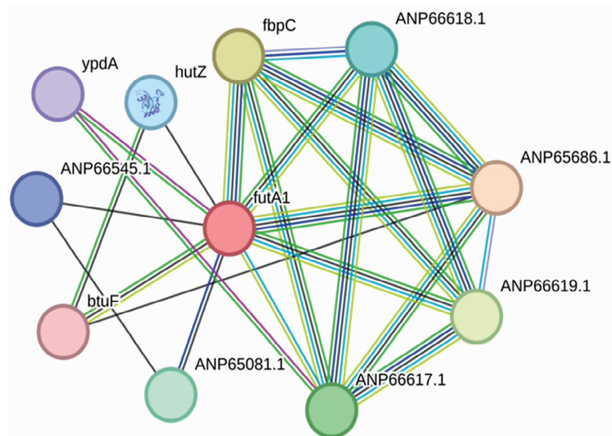


Fig. 8 FbpA protein interaction network

3 Discussion

V. alginolyticus, a primary pathogen responsible for vibriosis in marine aquaculture animals, can trigger large-scale epidemics by compromising host immune responses, leading to substantial economic losses. Current control strategies for vibriosis in China heavily rely on antibiotics. However, prolonged antimicrobial intervention has fostered the prevalence of drug resistance genes in *V. alginolyticus* and induced complex ecological risks, posing a severe threat to the sustainability of aquaculture and food safety[19]. Consequently, the development of subunit vaccines characterized by high immunogenicity and low biotoxicity has emerged as a prominent research focus in vaccinology.

Current research on *V. alginolyticus* predominantly centers on traditional pathogen isolation and identification, with emphasis on gene expression regulation, elucidation of pathogenic mechanisms, and host adaptation control mechanisms[20]. In contrast, systematic investigations into its virulence-associated genes remain relatively scarce. *V. alginolyticus* employs secreted virulence factors, including adhesins, lipopolysaccharide (LPS), type III secretion systems (T3SS), and outer membrane proteins, to facilitate host environment adaptation and regulate cellular invasion[21]. Li Shengjun *et al.* proposed that Listeria monocytogenes utilizes its surface protein FbpA to mediate host cell adhesion and invasion control, where FbpA acts as a pivotal adhesion receptor[22]. Therefore, in-depth research on virulence-associated genes of *V. alginolyticus*, screening for protective antigens, and establishing anti-infection target systems represent crucial pathways towards achieving breakthroughs in the immunoprophylaxis and immunotherapy of vibriosis.

Bioinformatics elucidates protein mechanisms through structural modeling and functional prediction[23]. Leveraging multi-dimensional predictive models encompassing protein evolutionary tracing, physicochemical properties, and secondary/tertiary structures enables precise epitope mapping, significantly enhancing antigen design efficiency and reducing experimental empiricism. This approach has been successfully integrated throughout the vaccine development pipeline, serving as a core technological pillar in modern vaccinology[24]. Liu Fengjuan *et al.* rapidly identified the *fbpA* gene (encoding the Ag85A antigen) in the Bacillus Calmette-Guérin (BCG) vaccine using single-colony PCR, confirming its capacity as a candidate protective antigen for Mycobacterium tuberculosis to elicit specific immune responses. The established prokaryotic expression system demonstrated both efficiency and cost-effectiveness[25].

This study conducted bioinformatic analysis based on the amino acid sequence of the FbpA protein. Signal peptide prediction indicated the presence of an N-terminal signal peptide in FbpA, leading to the inference that FbpA is a secretory protein. Transmembrane structure analysis revealed no transmembrane domains, further supporting its potential classification as a secreted or extracellular protein. Functional site prediction identified within the FbpA amino acid sequence: 2 N-glycosylation sites, 5 protein kinase C phosphorylation sites, 4 casein kinase II phosphorylation sites, 3 tyrosine kinase phosphorylation sites, 7 N-myristoylation sites, and 7 microbody C-terminal targeting signals. Protein phosphorylation, serving as a dynamic regulatory mechanism[26-27], acts as a molecular switch by modifying protein function and structure, governs cell cycle control, and is extensively involved in signal transduction across organisms. Notably, while *V. alginolyticus*, lacking eukaryotic-specific organelles, is incapable of performing glycosylation and acylation modifications on FbpA, its phosphorylation modification pathways remain intact[28]. This provides a feasible basis for subsequent vaccine development utilizing prokaryotic expression systems.

The rapid advancement of bioinformatics technology is driving innovation in antigen prediction and screening, establishing it as a core and critical technology in vaccine research and development[29-30]. Historically, in the quest for common *Vibrio* antigens, research emphasis on *V. alginolyticus* has largely focused on infection-related enzymes[31], adhesion/colonization factors, and transport proteins[32], whereas studies on the FbpA protein of *V. alginolyticus* remain relatively limited.

This study successfully cloned the virulence-associated *fbpA* gene from *V. alginolyticus* strain HY9901. Sequence analysis revealed that the amino acid sequence of the encoded FbpA protein is highly conserved, exhibiting over 70% similarity to other *Vibrio* species. Phylogenetic analysis demonstrated that the FbpA protein of *V. alginolyticus* HY9901 clusters distinctly within the same subclade as *Vibrio antiquarius* and the *Vibrio diabolicus* subgroup, indicating a close phylogenetic relationship among them. This finding is largely consistent with results from traditional classification based on morphological and biochemical characteristics, further validating the feasibility of utilizing the FbpA protein as a molecular marker for investigating interspecies evolutionary relationships within the Vibrio genus.

4 Conclusion

This study successfully amplified and sequenced the *fbpA* gene from *V. alginolyticus* HY9901. Sequence analysis revealed that the gene is 1 014 bp in length and encodes 337 amino acids. The theoretical pI (isoelectric point) is 5.97 and the theoretical molecular weight is 37.589 kDa. The protein lacks transmembrane domains, is hydrophobic, and harbors multiple functional sites. Phylogenetic tree construction based on the deduced *fbpA* amino acid sequence and those of other species indicated a close evolutionary relationship with *Vibrio antiquarius* and the *Vibrio diabolicus* subgroup. Prediction of FbpA functional domains identified one primary domain, Pfam. Secondary structure prediction demonstrated that the FbpA protein is composed of alpha-helices (49.85%), random coils (30.27%), extended strands (14.24%), and beta-sheets (5.64%).

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