Molecular Cloning and Bioinformatics Analysis of *cyaA* Gene of *Vibrio alginolyticus* Strain HY9901

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Abstract [**Objectives**] This study was conducted to explore the biological functions of *cyaA* gene of *Vibrio alginolyticus*. [**Methods**] With DNA of *V. alginolyticus* HY 9901 as a template, primers were designed according to the sequence of *cyaA* gene, and the *cyaA* gene was amplified by PCR. Bioinformatics analysis was performed. [**Results**] The *cyaA* gene of *V. alginolyticus* HY9901 was 2 529 bp in size, and encoded 842 amino acids. The molecular structure of CyaA protein was $C_{4\ 358}H_{6\ 745}N_{1\ 171}O_{1\ 286}S_{35}$. Its theoretical molecular weight was 97.241 67 kDa and the theoretical pI value was 5.56. It had no signal peptide and transmembrane domain. CyaA protein had three N-terminal glycosylation sites, one cAMP and cGMP-dependent protein kinase phosphorylation site, nine protein kinase C phosphorylation sites, nine casein kinase II phosphorylation sites, one tyrosine kinase phosphorylation site, seven N-terminal myristoylation sites, one pentenyl binding site and ten microbody C-terminal localization signal sites. Subcellular localization prediction showed that CyaA protein was mainly located in the nucleus and cytoplasm. Through multi-sequence alignment and phylogenetic tree construction, it was concluded that *V. alginolyticus* had high CyaA homology with other *Vibrio* species. *cyaA* of *V. alginolyticus* was clustered with *Vibrio fluminensis* and *Vibrio marinisedimini*, and they were closely related. The secondary structure of CyaA protein consisted of α-helixes (43.11%), random coils (38.00%) and extended strands (14.49%). In protein network interaction, it was found that the proteins adjacent to CyaA protein were Crp-2, CpdA, Crr, PtsG-2, ANP67209.1, Crp-1, PykF, Pyk, RelA and Ndk. [**Conclusions**] This study provides a new idea for formulating strategies for the prevention and control of vibriosis.

Key words *Vibrio alginolyticus*; *cyaA* gene; Bioinformatics analysis
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Vibrio alginolyticus, belonging to the genus *Vibrio* of Vibrionaceae, is a kind of thermophilic, halophilic and facultative anaerobic Gram-negative short rod-shaped bacterium[1-2], which is considered as the main pathogen of vibriosis in many marine organisms[3]. As a common conditional pathogen, *V. alginolyticus* will not cause harm to marine life under normal circumstances, but when the environment is harsh or the number of *V. alginolyticus* increases rapidly, it can colonize the intestinal tract, gill and epidermis of fish, resulting in the occurrence of vibriosis, which is reflected by skin ulceration, loss of appetite, anal swelling and other symptoms[4] and brings great losses to the aquaculture industry. Meanwhile, *V. alginolyticus* is a zoonotic bacterium, which can infect human body through direct invasion, thus causing wound infection, otitis media, septicemia and other diseases[5-6]. *V. alginolyticus* has many virulence factors, mainly including extracelluar products (ECPs), lipopolysaeeharides (LPSs), siderophore and adhesin factor[7], and the existence of virulence genes and the regulatory network formed by their interaction affect the virulence of *V. alginolyticus*.

The protein encoded by *cyaA* gene is an adenylate cyclase (AC)[8], which can invade eukaryotic cells and catalyze the synthesis of cyclic adenosine monophosphate (cAMP) after being activated by endogenous calmodulin[9-10]. cAMP is the second messenger involved in cell function regulation. cAMP and its signal transduction receptor CRP (cAMP receptor protein) combine to form a cAMP-CRP complex, which plays an important role in a series of catabolism, flagella synthesis, toxin production and other metabolic processes[11]. Under the condition of insufficient glucose supply, adenylate cyclase is activated, and catalyzes the production of cAMP molecules to form cAMP-CRP complex, which in turn activates specific gene programs, thus enabling *Escherichia coli* to use alternative carbon sources to maintain its life activities[12]. *cyaA* gene is related to the formation of type I fimbriae. After *cyaA* gene is deleted from sheep lung-derived *E. coli*, the synthesis of fimbriae and flagella is affected by cAMP-CRP complex, which makes it difficult for them to adhere, resulting in the inability to form a complete biofilm[13]. In the process of colonization of respiratory epithelial cells by *Bordetella pertussis*, adenylate cyclase can destroy the integrity of tight junctions, overcome epithelial barriers and establish a stable niche[14].

At present, many studies on *cyaA* gene are concentrated in *B. pertussis*, *E. coli* and *Salmonella*, while few reports have been conducted on *cyaA* gene and its related coding protein in *V. alginolyticus*. In this study, the *cyaA* gene in *V. alginolyticus* HY9901 was amplified by PCR, and its bioinformatics analysis was carried out to explore the specific role of *cyaA* in *V. alginolyticus* and

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provide new ideas for formulating strategies for preventing and controlling vibriosis.

Materials and Methods

Materials

Strain HY9901, a virulent strain of *V. alginolyticus*, was isolated and preserved from diseased *Lutjanus sanguineus* in Zhanjiang, Guangdong Province by our laboratory.

Main reagents ExTaq DNA polymerase was purchased from Takara, and bacterial genomic DNA extraction kit and DNA gel recovery kit were purchased from Tiangen Biotech Co. Ltd. PCR primer synthesis and sequencing were completed by Sangon Biotech(Shanghai) Co., Ltd.

Methods

Extraction of total DNA from *V. alginolyticus* HY9901 *V. alginolyticus* HY9901 was coated on TSA plates, and single colonies were inoculated in TSB (5% NaCl) medium, and cultured at 28 ℃ for more than 12 h. A proper amount of bacterial liquid was added into an EP centrifuge tube, which was centrifuged at 10 000 rpm/min for 1 min to collect bacteria. Genomic DNA was extracted according to instructions of the kit, and stored at −20 ℃ for later use.

Cloning of *cyaA* gene According to the sequence of *cyaA* gene of *V. alginolyticus*, a pair of primers was designed. The forward primer was TTGCAGGCTTACACCCAAAAAATC, and the reverse primer was TTACGCGTTCACAGTTTTGGTCGGG. With the extracted total DNA of *V. alginolyticus* HY9901 as a template, PCR was started with pre-denaturation at 94 ℃ for 5 min, followed by 35 cycles of 94 ℃ for 30 s, 55 ℃ for 30 s and 72 ℃ for 40 s, and then extension at 72 ℃ for 10 min. The PCR products were detected by 1% agarose gel electrophoresis, and then the DNA gel recovery kit was used to cut the gel for recovery. The recovered fragment was cloned into pMD18-T vector and sent to the company for sequencing.

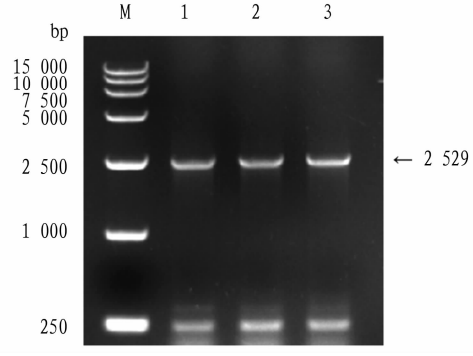
Bioinformatics analysis of *cyaA* from *V. alginolyticus* HY9901

Sequence homology alignment and similarity analysis were carried out by NCBI (http://blast.ncbi.nlm.nih.gov/blast.cgi). DNAMAN Version 6.0 (Lynnon Biosoft) was used for homology alignment analysis of amino acids. ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and EXPASY Proteomics Server (http://ca.expasy.org) were used for deducing the amino acid sequence, determining open reading frame (ORF) and calculated molecular weight (Mw) and predicting theoretical isoelectric point (pI). The sequences of signal peptides were predicted by online analysis software http://www.cbs.dtu.dk/services/SignalP. TMHMM Server 2.0 (http://www.cbs.dtu.dk/services/TMHMM) was adopted for prediction of transmembrane domains. SoftBe-rry-Psite (http://linux1.softberry.com/berry.phtml?topic=psite&group=programs&subgroup=proloc) was adopted for predicting distribution of functional sites in the amino acid sequence. Protein structural and functional domains were analyzed using InterProScan Sequence Search (http://www.ebi.ac.uk/Tools/InterProScan). Subcellular localization prediction was performed using PSORT II Prediction (http://psort.hgc.jp/form2.html). A phylogenetic tree was constructed by the neighbor-joining method using Clastal 2.0 and MEGA 5.0 software. Modeling was conducted using the SWISS-MODEL[19] (http://www.swissmodel.expasy.org/) program of ExPASy server, and 3D structural analysis software PyMOL Viewer was used for analysis.

Results and Analysis

Amplification of *cyaA* gene

cyaA gene was amplified by PCR. The amplification products were analyzed by agarose gel electrophoresis, and a specific band of about 2 529 bp was observed (Fig. 1). Sequencing of the amplification products and the cloning vector pMD18-T showed that *cyaA* gene contained an open reading frame of 2 529 bp, encoding 842 amino acids.



M: DL15000 DNA marker; lanes 1-3: cyaA PCR products.

Fig. 1 Amplification of cyaA gene

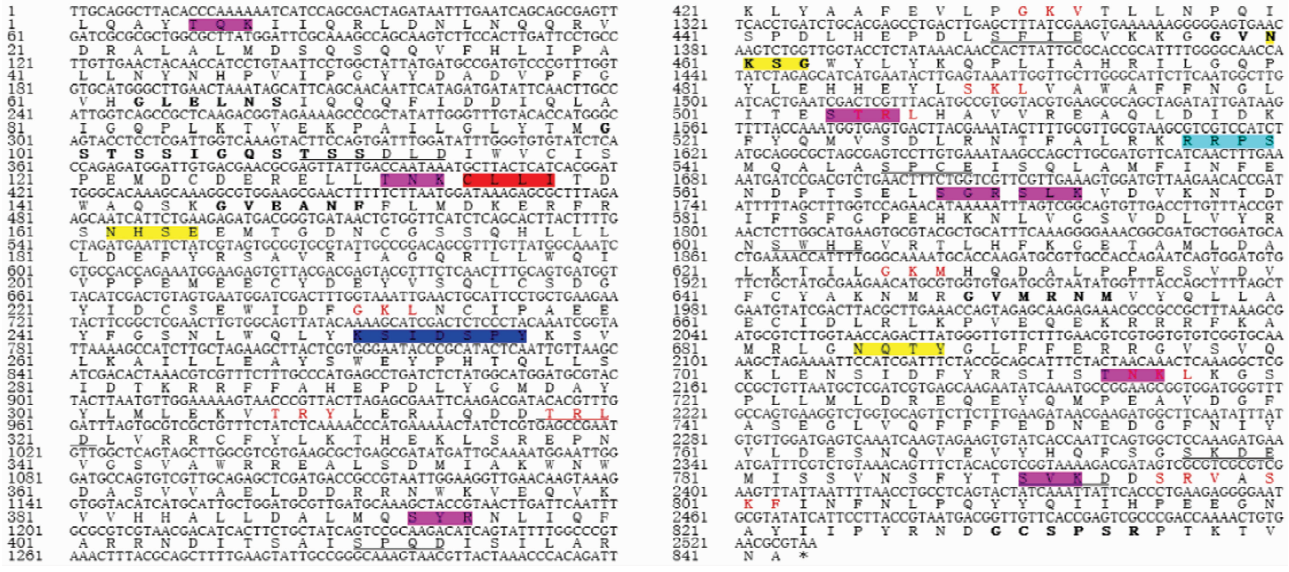
Physical and chemical properties of CyaA

CyaA protein of *V. alginolyticus* HY9901 was analyzed by ExPASy software. The results showed that the total number of atoms was 13 595, and the molecular structure was $C_{4\,358}H_{6\,745}N_{1\,171}O_{1\,286}S_{35}$. Its theoretical molecular weight was 97.241 67 kDa and the theoretical pI value was 5.56. The instability coefficient was 43.04 (unstable). The fat coefficient was 88.46, and the total average hydrophilicity was −0.356. The protein was hydrophobic overall. It contained no pyrrolysine (Pyl) and selenocysteine (Sec), and the total number of acidic amino acids (Asp + Glu) was 113, and the total number of basic amino acids (Arg + Lys) was 92. The molar extinction coefficient at 280 nm was 130 360 (mol/cm). The N terminal was leucine (Leu). The half life of culture and expression in mammalian reticulocytes in vitro was 5.5 h; the half life of expression in yeast was 3 min; and the half life of expression in *E. coli* was 2 min.

Sequence analysis of CyaA

The structure of signal peptides at the N terminal of CyaA amino acid sequence was predicted using the program of SignalP 4.0 Server, and it was found that there was no obvious signal peptide cleavage site and no signal peptide existed. It was predicted by the program of TMHMM Server 2.0 that the protein had no transmembrane domain. The program of SoftBerry Psite predicted following sites: three N-terminal glycosylation sites (162 − 165 aa, 460 − 463 aa, 685 − 688 aa), one cAMP and cGMP-dependent

protein kinase phosphorylation site (537 – 540 aa), nine protein kinase C phosphorylation sites (5 – 7 aa, 132 – 134 aa, 283 – 285 aa, 393 – 395 aa, 504 – 506 aa, 568 – 570 aa, 571 – 573 aa, 714 – 716 aa, 791 – 793 aa), nine casein kinase II phosphorylation sites (109 – 112 aa, 111 – 114 aa, 318 – 321 aa, 411 – 414 aa, 450 – 453 aa, 546 – 549 aa, 602 – 605 aa, 777 – 780 aa, 791 – 794 aa), one tyrosine kinase phosphorylation site (251 – 257 aa), seven N-terminal myristoylation sites (63 – 68 aa, 100 – 105 aa, 106 – 111 aa, 146 – 151 aa, 458 – 463 aa, 649 – 654 aa, 830 – 835 aa), one pentenyl binding site (135 – 138 aa), and ten microbody C – terminal localization signal sites (231 – 233 aa, 308 – 310 aa, 318 – 320 aa, 431 – 433 aa, 489 – 491 aa, 505 – 507 aa, 626 – 628 aa, 715 – 717 aa, 796 – 798 aa, 800 – 802 aa). The results of subcellular localization prediction in protein showed that CyaA was the most likely to be located in the nucleus and cytoplasm with a probability of 43.5%, and might be located in mitochondria with a probability of 8.7%, and in vacuoles with a probability of 4.3% (Fig. 2).



The terminator is indicated by ＊; the yellow parts are N-terminal glycosylation sites; the blue green part is the phosphorylation site of cAMP and cGMP-dependent protein kinase; the purple parts are the phosphorylation sites of protein kinase C; the underlined marks are the phosphorylation sites of casein kinase II; the blue part is the tyrosine kinase phosphorylation site; the bold font parts are the N-terminal myristoylation sites; the red part is the isopentenyl binding site; the red font parts are the microbody C-terminal localization signal sites.

Fig. 2 *cyaA* gene nucleotides and its encoded amino acid sequence

V. alginolyticus; *V. antiquarius* (WP_301657608.1); *Vibrio chemaguriensis* (WP_193448300.1); *Vibrio diabolicus* (WP_150915982.1); *Vibrio parahaemolyticus* (WP_025518361.1).

Fig. 3 Homology comparison of deduced amino acid sequence of *cyaA* gene

Homology and evolutionary analysis of CyaA

Through the analysis of the protein sequence of *V. alginolyticus* CyaA by BLAST, it was found that the CyaA protein sequence of *V. alginolyticus* had high homology with those of other *Vibrio* species. Among them, *Vibrio antiquarius* had the highest homology with *V. alginolyticus*, and their similarity was 98.22% (Fig. 3).

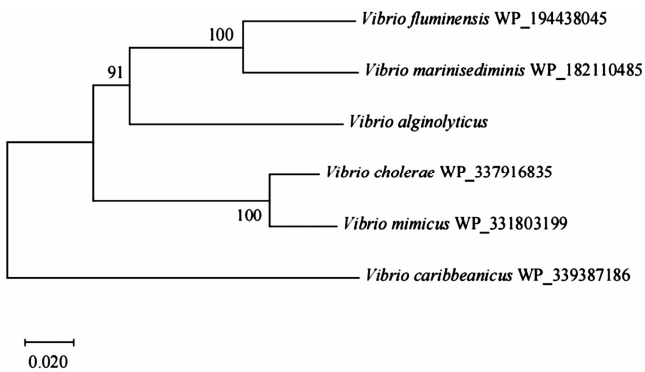


Fig. 4 Phylogenetic tree of CyaA amino acid sequence based on NJ method

A phylogenetic tree was constructed by the Neighbor-joining method in MEGA 5.0 software using the amino acid sequence of CyaA and other microorganisms. The results showed that cyaA of

V. alginolyticus HY9901 was clustered with *Vibrio fluminensis* and *Vibrio marinisediminis*, indicating that they were closely related (Fig. 4).

Functional domain, secondary and tertiary structure prediction of CyaA

According to the prediction by SMART program, it was found that there was a low complexity region (LCR) of protein located at 100-111 aa (Fig. 5). In the secondary structure prediction, alpha helixes accounted for 43.11%; random coils accounted for 38.00%; extended strands accounted for 14.49% (Fig. 6).

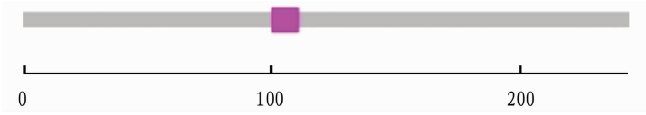
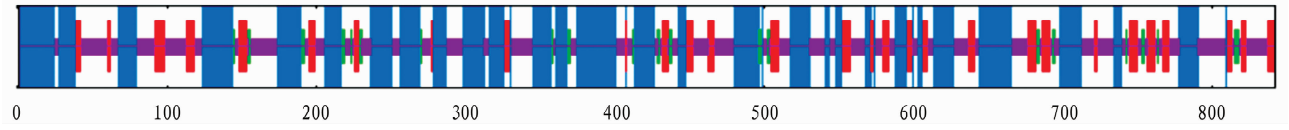


Fig. 5 Functional domain of CyaA



Blue: Alpha helix; purple: random oil; red: extended strand.

Fig. 6 Secondary structure prediction of CyaA

The amino acid sequence of CyaA was submitted to the SWISS-MODEL program, and homologous proteins were automatically searched as templates. The tertiary structure model of CyaA was obtained with the template of A0A3A2HWP3.1.A, and the consistency was 91.81% (Fig. 7).

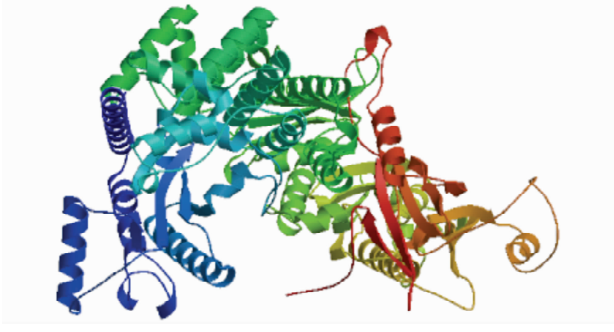


Fig. 7 Tertiary structure prediction of CyaA

Protein network interaction of CyaA

In protein network interaction, it could be found that the proteins adjacent to CyaA protein were Crp-2, CpdA, Crr, PtsG-2, ANP67209.1, Crp-1, PykF, Pyk, RelA and Ndk.

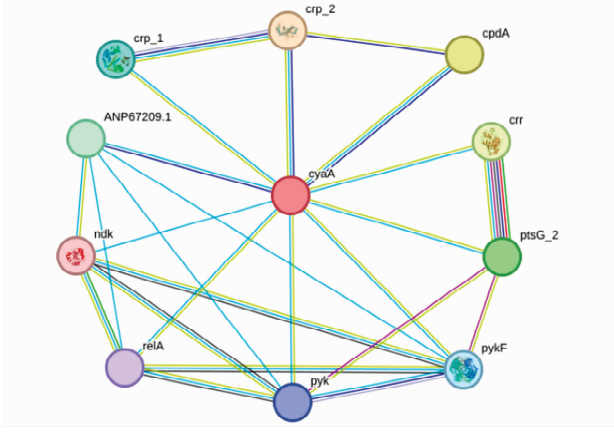


Fig. 8 Protein network interaction of CyaA

Conclusions and Discussion

Vibrio is a type of pathogenic bacteria that seriously harms aquaculture, and vibriosis caused by *V. alginolyticus* is one of the common diseases in marine aquaculture at present[15]. *cyaA* gene encodes adenylate cyclase, which catalyzes the synthesis of cAMP, and then plays a key role in many physiological processes through cAMP-CRP complex. In order to explore the biological function of *cyaA* gene of *V. alginolyticus*, *cyaA* gene of *V. alginolyticus* HY9901 was amplified in this study, with a size of 2 529 bp. Its biological information was analyzed, and the results show that the molecular structural formula of CyaA protein is $C_{4\,358}H_{6\,745}N_{1\,171}O_{1\,286}S_{35}$, which contains no signal peptide and transmembrane domain, and is not classified to membrane protein or secretory protein. CyaA protein has multiple functional sites, including three N-terminal glycosylation sites, one cAMP and cGMP-dependent protein kinase phosphorylation site, nine protein kinase C phosphorylation sites, nine casein kinase II phosphorylation sites, one tyrosine kinase phosphorylation site, seven N-terminal myristoylation sites, one pentenyl binding site and ten microbody C-terminal localization signal sites. Through multi-sequence alignment and phylogenetic tree construction, it is concluded that *V. alginolyticus* has high CyaA homology with other *Vibrio* species. It could be inferred that the protein may be an effective antigen against vibriosis, and it shows that cyaA of *V. alginolyticus* is closely related to *V. fluminensis* and *V. marinisediminis*.

Functional domain prediction of CyaA protein show that there is a low complexity region (LCR) in the center of the sequence. Previous studies have shown that proteins containing LCRs often have more binding partners on different protein networks, and LCRs in different positions in sequences have different functions. Proteins with LCRs in the center of sequences play an indispensable role in transcription and transcription regulation[16]. CyaA protein is an adenylate cyclase, and cAMP synthesized by it is the key molecule to regulate the reading of stop codon and the heterogeneity of rRNA expression. When the *cyaA* gene is knocked out, the catabolism of amino acids and the production of ATP will be first blocked, which will in turn significantly inhibit the transcription process of rRNAs and tRNAs[17], which is consistent with the predicted results. Protein interaction is the main way for protein molecules to exert their biological functions, and the construction of a protein interaction network is helpful to identify molecules that interact with the target protein, which provides an important basis

for studying the biological functions of the target protein and its mechanism of exerting biological effects[18]. Regmi[19] found that cAMP-CRP was an activator of metabolism, movement, capsule production and biological membrane formation of *Vibrio parahaemolyticus* by constructing deletion strains of *crp* gene and *cyaA* gene. In this study, the prediction results of protein network interaction show that CyaA protein interacts with Crp-1 and Crp-2 proteins, which is consistent with related contents described by Regmi above, suggesting that CyaA protein may have similar biological functions in *V. alginolyticus*, which still needs to be further studied through related experiments.

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