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研究报告

Characterization of *AcrA* gene from *Vibrio alginolyticus* Strain HY9901

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Abstract: A 460 bp internal fragment of the *AcrA* gene from *Vibrio alginolyticus* strain HY9901 was amplified by PCR with designed primers and the unknown flanking sequence of 5′- and 3′- ends of the *AcrA* gene was finally characterized by inverse PCR and nested PCR. Sequence analysis showed the *AcrA* gene contained 1101 bp ORF encoding 366 amino acids and the deduced amino acid sequence of the precursor from *Vibrio alginolyticus* strain HY9901 showed significant homology with the putative protein of other *Vibrio* species. The AcrA shows 76%, 73%, 71% and 70% homology with *V. vulnificus* strain YJ016, *V. parahaemolyticus* strain RIMD 2210633, *V. splendidus* strain 12B01 and *V. cholerae* O1 biovar eltor str. N16961 respectively.

Keywords: *Vibrio alginolyticus*, *AcrA* gene, Gene cloning

溶藻弧菌 HY9901 *AcrA* 基因克隆与序列分析

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摘 要: Touchdown PCR 扩增溶藻弧菌 HY9901 *AcrA* 基因部分序列, 得一 460 bp 片段, 再以反向 PCR 和巢式 PCR 联合扩增其侧翼序列, 拼接得一由 1101 nt 组成, 共编码 366 aa 的完整基因。该基因演绎的氨基酸序列与几种弧菌的同源性都比较高, 与创伤弧菌 YJ016、副溶血弧菌 RIMD 2210633、灿烂弧菌 12B01、霍乱弧菌 O1 N16961 同源性分别为 76%、73%、71%和 70%。

关键词: 溶藻弧菌, *AcrA* 基因, 基因克隆

1 Introduction

Vibrio alginolyticus, a halophilic *Vibrio* species, has been reported to be responsible for extensive outbreaks of the ulcer disease of the gilthead sea bream *Sparus aurata*, turbot *Scophthalmus maximus*, Crimson snapper *Lutjanus erythopterus* (Bloch), sea mullet *Mugil cephalus* and rotifer *Brachionus plocatilis* in the

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world[1,2,3]. With the contamination of circumstance, the *V. alginolyticus* strains isolated in the past were found to resistant to several antibiotics. However, there is paucity of information on the genetic basis of drug resistance in these strains. Antibiotic resistance arises through overexpression of efflux systems and can be mediated by plasmids, integrons and transposons[4,5,6]. In those studies, Vibrios cell obtains genes for a multidrug efflux pump or a silent gene for the pump becomes functional, the cell becomes resistant to many drugs. Thus, the presence of functional multidrug efflux pumps in Vibrios cells is a serious problem for the treatment of aquatic animals infected with such Vibrios[7,8]. In the past decade, a number of multiple drug resistant efflux pump have been characterized in *V. cholerae*, *V. parahaemolyticus* and *V. fluvialis*[9,10,11]. Well-studied examples of Vibrios multiple drug resistant efflux pumps have indicated that the AcrA is a subunit made up of the AcrAB-TolC pump[12,13]. AcrAB-TolC appears to be the main pump providing intrinsic resistance to low levels of many toxic compounds in nature[14,15]. The gene cloning and characterization of AcrA were reported in Vibrios except *V. alginolyticus*, such as *V. parahaemolyticus*, *V. vulnificus*, *V. cholerae* and *V. splendidus*[9,10,11,16,17]. Here we report the gene cloning and characterization of AcrA in *V. alginolyticus*.

The objective of this study was to characterize the complete nucleotide sequence of the *AcrA* of *V. alginolyticus*, using the sequences of the highly conserved DNA area of the *AcrA* gene among Vibrios as starting points. The lack of detailed information on the *AcrA* of *V. alginolyticus* makes the study difficult. Study on the molecular basis of drug efflux is significative for the development of modulators that inhibit multidrug efflux pumps through high affinity binding.

2 Materials and methods

2.1 Strains and plasmids

V. alginolyticus strain HY9901 was isolated from the diseased marine-cultured fish *Lutjanus sanguineus* (Cuvier) at Zhanjiang, and stored at our laboratory. *Escherichia coli* DH5α was used as the host strain for transformation. *E. coli* plasmid PMD18-T was used as a cloning vector.

2.2 Extraction of genomic DNA

V. alginolyticus strain HY9901 was grown in tryptic soy broth (TSB) containing 2% NaCl for 4 h at 28℃. Genomic DNA was isolated according to the method of Marmur, followed by standard phenolic extraction[18].

2.3 Cloning of partial *AcrA* gene

Part of the *AcrA* gene of *V. alginolyticus* was initially amplified by PCR based on the sequences of the highly conserved DNA area of the *AcrA* gene among Vibrios. A pair of specific primers was designed (G1: 5′-GGAGTCAAACAGCCACCG-3′; G2: 5′-TGGGCAAAGATACCACATCG-3′). PCR was performed at 94℃ for 5 min and then 1 min at 94℃, the annealing temperature was 60℃ for the first cycle, decreasing by 1℃ for each of the next 10 cycles until 50℃ 1 min; followed by 25 cycles with an annealing temperature of 50℃, and 1.5 min at 72℃ for 35 cycles followed by incubation at 72℃ for 10min. The PCR product was purified from 1% agarose gel using EZ-10 Spin Column Gel Extraction Kit and cloned into PMD18-T vector, and then sequenced at Shanghai Sangon Biologic Engineering&Technology and Service Co.Ltd.

2.4 Digestion and ligation of genomic DNA from *V. alginolyticus* strain HY9901

The genomic DNA of *V. alginolyticus* strain HY9901 was divided into four groups, and each group was digested with one of the following restriction enzymes overnight: *Eco*RⅠ, *Pst*Ⅰ, *Sac*Ⅰ, *Hin*dⅢ. The endonucleases were then thermally inactivated at 65℃ for 20 min, and the DNA digested by the five groups was ligated into circular DNA with T4 DNA ligase overnight at 16℃ respectively. The resulting self-ligated DNA was precipitated with ethanol and resuspended in sterile distilled water.

2.5 Cloning of the flanking *AcrA* gene by inverse PCR and nested PCR

According to PCR product of the partial *AcrA* gene sequence before, two pairs of primers were designed: H1: 5′-TCACTTTAACCGTGCCGTTTA-3′; H2: 5′-GCCCAGGTAGACAGGACGAA-3′, for inverse PCR; I1: 5′-CGCCAAACCCACGCAAAG-3′; I2: 5′-GGAGGCCCTGATAGTGATTGT-3′, for nested PCR. The reaction mixture of inverse PCR was added 4% Dimethyl Sulfoxide(DMSO). The touchdown PCR program was used: denaturation at 94℃ for 5 min, followed by 35 cycles: 1min at 94℃, the annealing temperature was 60℃ for the first cycle, decreasing by 1℃ for each of the next 8 cycles until 53℃ 1min; followed by 25 cycles with an annealing temperature of 53℃, 2 min at 72℃, followed by 10min at 72℃. The product of inverse PCR was diluted to 200 folds and 1μL dilution was used as the DNA sample for the nested PCR. The reaction mixture of nested PCR was added 0.1% Triton X-100 and performed by routine condition. The PCR product was purified and cloned into PMD18-T vector as above, then sequenced.

2.6 Computer sequence analysis

Nucleotide sequence data were compiled and assemble using the Seqman of Lasegene® software package (DNASTAR, Madison, WI, USA). Database searches were performed using BlastX (http: //www.ncbi.nlm.nih.gov/blast). Open reading frames(ORFs) were identified using ORF Finder (http: //www.ncbi.nlm.nih.gov/gorf/gorf.html). Signal sequence predication was performed using SignalP (http: //www.cbs.dtu.dk/services/SignalP). Protein masses and isoelectric points were determined online, using ProtParam tools (http://us.expasy.org/tools/protparam.html). Proteins were examined for conserved motifs using Pfam(http://pfam.wustl.edu/hmmsearch.shtml).

3 Results and discussion

3.1 Cloning of the partial *AcrA* gene and flanking *AcrA* gene

An approximately 460 bp PCR product was obtained when PCR amplification of the *AcrA* gene of *V. alginolyticus* strain HY9901 was performed using primers G1 and G2. The sequence analysis showed the PCR product contains an incomplete ORF and revealed extensive homology(85% indentity) with *AcrA* gene of *V. vulnificus* strain CMCP6 in the EMBL/GenBank Data Library(AE016795). Successive inverse PCR and nested PCR experiments allowed amplification of the full length *AcrA* gene. Self-ligated circular DNA derived from *Pst*I-digested *V. alginolyticus* strain HY9901 chromosomal DNA was used as a template to finally amplify an approximately 2.5 kb fragment. This result of sequences indicated *Pst*I was proper restriction endonuclease digesting the chromosomal DNA as the template of inverse PCR.

The flanking sequence of *AcrA* gene was ready to amplify by conventional inverse PCR, but subsequently agar gel electrophoresis showed the amplified fragment was none. DMSO may decrease self anneal of primers of PCR system, hence by adding 4% DMSO to the inverse PCR system the amplified fragment could been seen on the agar gel electrophoresis[19,20].

3.2 Characteristic of *AcrA* gene

The sequences were then aligned and assembled obtaining a final sequence to comprise a complete ORF, the *AcrA* gene, which from the presume puptive start codon (ATG) to the stop coden (TAA) contains 1101 nt encoding 366 aa residues (Fig. 1). The *AcrA* gene has been deposited with GenBank accession no. DQ440528 and would encode the protein with molecular weight of 40.3 kD and PI of 5.66. Computer analysis of the deduced protein product of *AcrA* gene with the Blast program identified similarities with the putative protein of some Vibrios. The AcrA shows 76%, 73%, 71% and 70% homology with *V. vulnificus* strain YJ016, *V. parahaemolyticus* strain RIMD 2210633, *V. splendidus* strain 12B01, *V. cholerae* O1 biovar eltor str. N16961(Fig. 2). Compared with the homology of AcrA reaching 95% in Escherichias, that is very lower in *Vibrios*, As to the reason needs further clarification.

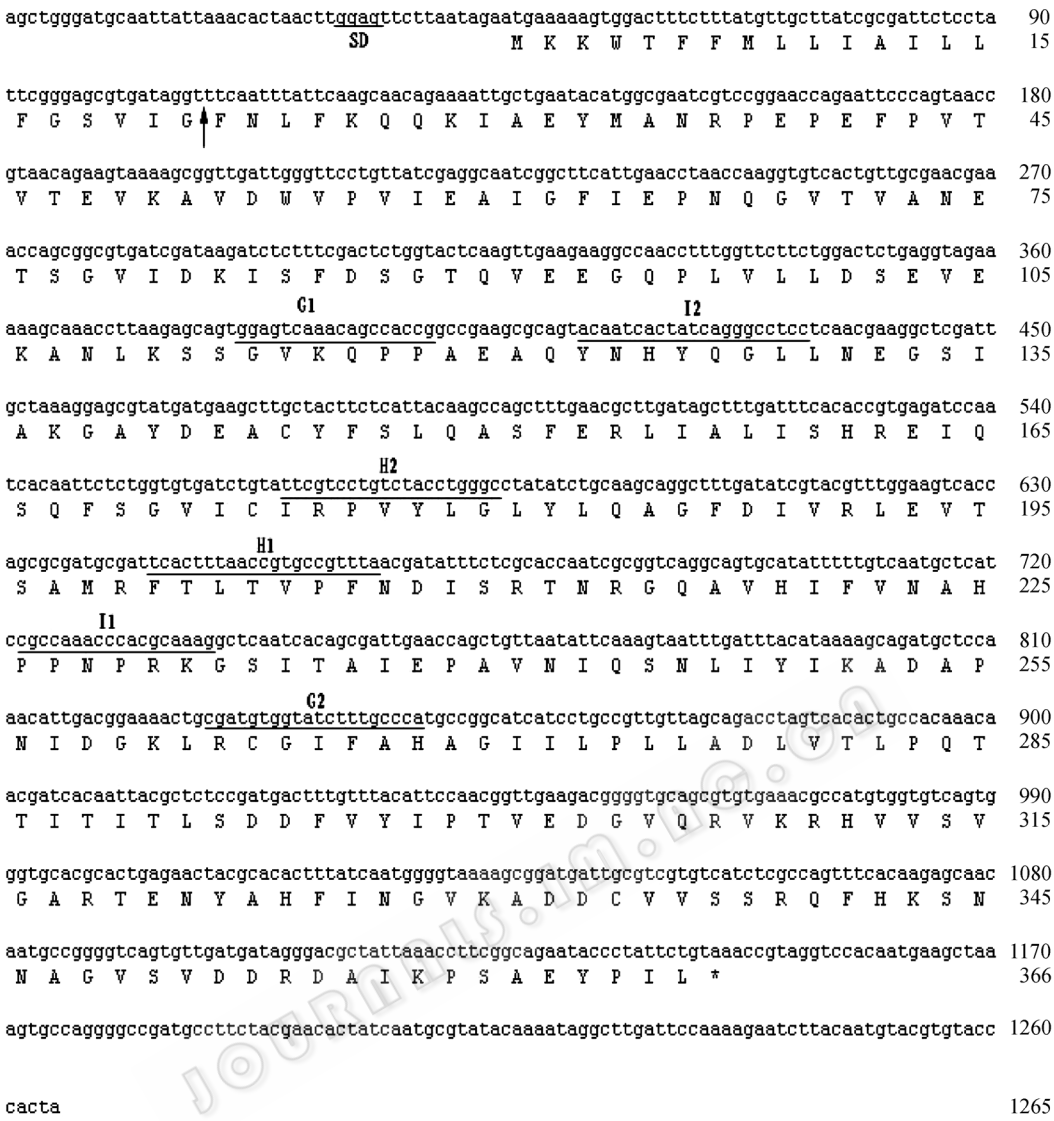

Fig. 1 The nt sequence and deduced aa sequence of *AcrA* gene.

The cleavage site for N-signal peptide is indicated by an arrow (↑). The Shine-Dalgarno (SD) ribosome binding site is indicated. An asterisk (*) marks the stop condon. The nucleotide sequences used to design PCR primers are underlined.

The putative Shine-Dalgarno sequence, GGAG, was found at 11 nucleotides upstream from the ATG codon. A nucleotide sequence of dyad symmetry wasn't found in the downstream region of the ORF. The deduced amino acid of the putative signal peptide sequence of 21 residues at the N-terminus sequence suggest AcrA could be secreted, which is very important for the activity of AcrA.. This result suggests that the protease was expressed as a precursor form, which includes a pre- and pro-sequences. The putative signal peptide sequence of the AcrA has the characteristics of a typical bacterial signal sequence with an N-terminal positive charged stretch followed by some hydrophobic residues, a helix-breaking praline, and a typical Gly-X-Phe signal peptidase cleavage site[21]. Type motif HlyD is observed from site 68 to 111 in the deduced amino acid sequence.

The present study indicates that the *AcrA* gene existed in *V. alginolyticus*. The widely used antibiotics may activate the AcrAB-TolC efflux pump, which in return results in stronger multiple drug resistant than before. This study will lead to a better understanding in the molecular mechanism of multiple drug resistant of *V. alginolyticus*.

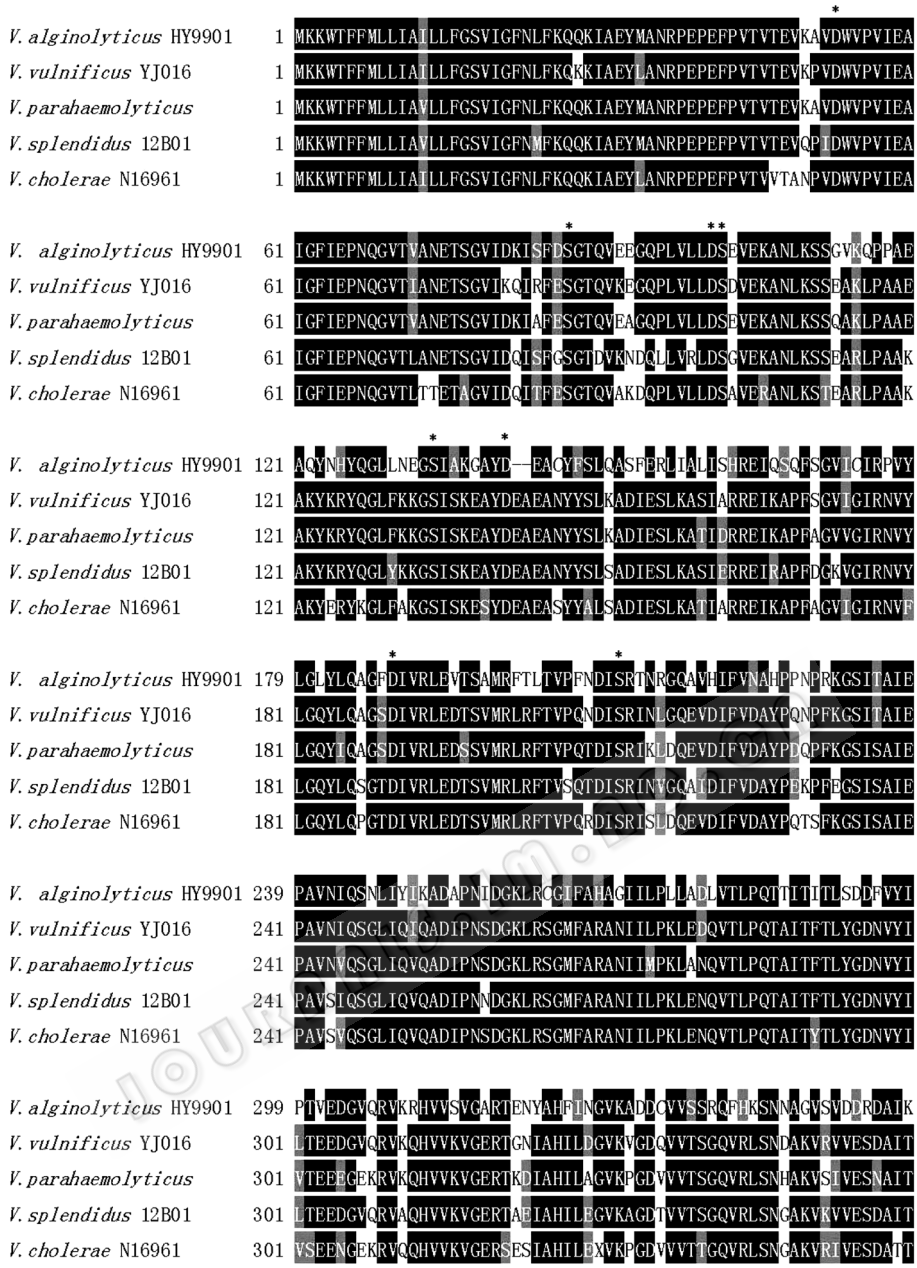

Fig. 2 Alignment of the deduced aa sequence of *V. alginolyticus* HY9901, *V. vulnificus* YJ016, *V. parahaemolyticus* RIMD 2210633, *V. splendidus* 12B01 and *V. cholerae* O1 biovar eltor str. N16961. The asterisks (*) mark the active sites.

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