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

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Abstract [**Objectives**] This study was conducted to understand the structure and function of MsrA protein. [**Methods**] With *Vibrio alginolyticus* HY9901 as the object of study, primers were designed to amplify the full-length gene of *msrA*, and its bioinformatics analysis was carried out. [**Results**] The full length of *msrA* gene was 639 bp, encoding 212 amino acids, and its theoretical molecular weight was about 23 729.60 Da. The protein had a stable structure, and it was hydrophobic overall. The structure of signal peptides at the N terminal of the amino acid sequence was predicted, and it was found that there was no signal peptide cleavage site and no transmembrane region. The amino acid sequence of MsrA contained multiple signal binding sites. Protein subcellular localization showed that MsrA protein was most likely located in the cytoplasm. Homology analysis showed that MsrA of *V. alginolyticus* had high homology with other *Vibrio* species, and the highest homology with *V. alginolyticus*. In the prediction of functional domains, MsrA had the function of methionine sulfoxide reduction. In secondary structure prediction, MsrA contained random coils at a proportion of 46.70%, which was the highest. The similarity between the tertiary structure model of MsrA and template Q87SW6.1.A was 89.15%. PTM analysis showed that MsrA protein had many PTM modification sites such as phosphorylation and glycosylation sites. [**Conclusions**] This study provides some reference value for further study on the role of MsrA in bacterial antioxidant stress.

Key words *Vibrio alginolyticus*; Gene cloning; MsrA; Bioinformatics analysis

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Vibrio alginolyticus is a kind of common pathogenic microorganism in seawater, which can infect aquatic organisms such as fish, crustaceans and molluscs and cause diseases[1]. The bacterium is short rod-shaped and has polar single flagella. It is mostly distributed in water bodies with high salinity such as oceans and estuaries[2]. *V. alginolyticus* is a facultative anaerobic conditional pathogen, which can rapidly proliferate into a dominant community when the living conditions are suitable, and then infect hosts quickly and efficiently under the action of various virulence factors, causing diseases[3]. In addition, this bacterium is zoonotic, and can infect humans and cause many diseases such as food poisoning, otitis media, conjunctivitis and septicemia, posing a serious threat to human health[4-7].

Methionine (Met), as a multifunctional amino acid, is easily oxidized by ROS, resulting in two diastereomer products, methionine-S-sulfoxide (Met-S-SO) and methionine-R-sulfoxide (Met-R-SO), which inactivate the function of protein[8]. Methionine sulfoxide reductase (Msr) is an enzyme that can repair methionine oxidative damage, and it participates in the defense against oxidative stress by reducing methionine sulfoxide residues in protein[9]. The enzyme mainly includes two types: methionine sulfoxide reductase A (MsrA) and methionine sulfoxide reductase B (MsrB), which specifically catalyze the reduction of free or protein-bound Met-S-SO and Met-R-SO to Met, respectively, so as to restore the normal physiological function of protein[10-11]. However, the efficiency of MsrA is much higher than that of MsrB in repairing the oxidation of free Met, and MsrB is growth-dependent[12-13]. Previous studies have shown that MsrA plays a very important role in the process of bacteria's anti-oxidative stress. It can not only directly repair Met oxidation, but also act as a free radical sink as a target of oxidative stress, thus protecting other amino acids from damage[14-15].

Although the antioxidant effect of MsrA in bacteria has been studied to some extent, the mechanism of MsrA participating in oxidative stress defense in *V. alginolyticus* is relatively less studied. In this study, the *msrA* gene of *V. alginolyticus* HY 9901 was cloned and analyzed by bioinformatics, and the structure and function of MsrA protein were preliminarily understood, providing some reference value for further study on its role in bacterial antioxidant stress.

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[16].

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

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primer synthesis and sequencing were completed by Sangon Biotech(Shanghai) Co., Ltd. The concentration of antibiotics ampicillin (Amp) was 100 μg/ml.

Methods

Extraction of total DNA from *V. alginolyticus* HY9901 Single colonies of *V. alginolyticus* HY9901 were isolated, and cultured at 28 ℃ with oscillation. A proper amount of bacterial liquid was centrifuged to collect bacteria. Genomic DNA was extracted according to instructions of the kit, and stored at −20 ℃ for later use.

Cloning of *msrA* gene According to the sequence of *msrA* gene of *V. alginolyticus*, a pair of primers was designed (Table 1). 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 30 cycles of 94 ℃ for 30 s, 50 ℃ 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.

Table 1 Primers for gene cloning

Primer name	Primer sequence
msrA-F	ATGCTCAATAAACAAACA
msrA-R	TTATCCTTGGAGGCTTG

Bioinformatics analysis of MsrA from *V. alginolyticus* HY9901 Sequence homology alignment and similarity analysis were performed by NCBI[17]. Amino acid homology alignment analysis was carried out by DNAMAN software. ExPASy Proteomics Server was employed for deducing the amino acid sequence and calculated molecular weight (Mw) and predicting theoretical isoelectric point (pI)[18]. Signal peptides, transmembrane domains, subcellular localization, functional sites and post-translational modification sites of the protein were predicted by online software[19-21]. InterProScan was adopted to analyze functional domains of protein structure. A phylogenetic tree was constructed using Clastal 2.0 and MEGA 5.0 software[22]. 3D modeling of protein tertiary structure was carried out by SWISS-MODEL[23] program. STRING database was searched for protein-protein network interaction diagrams[24].

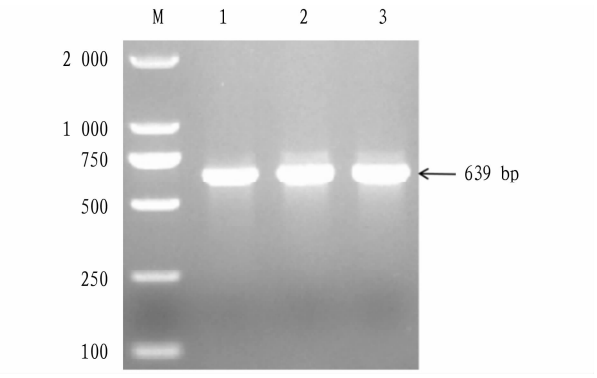
Results and Analysis

Full-length cloning of *msrA* gene

After PCR amplification and agarose gel electrophoresis analysis, a single band with a size of about 639 bp was obtained (Fig. 1). It was confirmed by sequencing to be a fragment of *msrA* gene, encoding 212 amino acids.

Analysis of physical and chemical properties

MsrA protein of *V. alginolyticus* HY9901 was analyzed by ExPASy software. The results showed that the total number of atoms was 3 285, and the molecular structure was $C_{1\,056}H_{1\,613}N_{283}O_{325}S_8$. Its theoretical molecular weight was 23 729.60 Da and the theoretical pI value was 5.20. The instability coefficient was 39.32, indicating that the protein was stable. The fat coefficient was 73.58, and the total average hydrophilicity was −0.471. Therefore, the protein is a hydrophobic protein overall. It contained no pyrrolysine (Pyl) and selenocysteine (Sec). The molar extinction coefficient at 280 nm was 34 630 $M^{-1} \cdot cm^{-1}$. The total number of acidic amino acid residues (Asp + Glu) was 24, and the total number of basic amino acids (Arg + Lys) was 36. The N terminal was methionine (Met). The half lives of expression in yeast and *E. coli* were 20 and 10 h, respectively; and the half-life of *in-vitro* culture and expression in mammalian reticulocytes was 30 h.



Lane M: DL2000 DNA Marker; Lanes 1 − 3: PCR products of *msrA* gene.

Fig. 1 Amplification of *msrA* gene

Analysis of MsrA sequence

The signal peptide structure at the N terminal of amino acid sequence of *msrA* gene was predicted by SignalP 5.0 Server program, and it was found that there was no obvious signal peptide cleavage site and no signal peptide existed. The TMHMM Server 2.0 program predicted that the protein had no transmembrane helix structure.

SoftBerry-Psite program predicted that MsrA amino acid sequence contained two N-glycosylation sites, one protein kinase C phosphorylation site, two casein kinase II phosphorylation sites, six N-myristoylation sites, one prokaryotic membrane lipoprotein-lipid attachment site, one isopentenyl binding site, and one microbody C-terminal target signal (Fig. 2). The prediction of protein subcellular localization showed that MsrA was most likely located in the cytoplasm.

Homology and evolutionary analysis

According to the results of BLAST alignment, homology analysis was conducted with DNAMAN software. It was found that MsrA of *V. alginolyticus* had high homology with MsrA of other *Vibrio* species. The homology with MsrA of *V. antiquarius* was highest, and their similarity was 85.96% (Fig. 3).

A phylogenetic tree was constructed by the Neighbor-joining method in MEGA 5.0 software using the deduced amino acid sequence of MsrA and other *Vibrio* species. The results showed that MsrA of *V. alginolyticus* HY9901 and *Vibrio diabolicus* belonged to the same subgroup (Fig. 4).

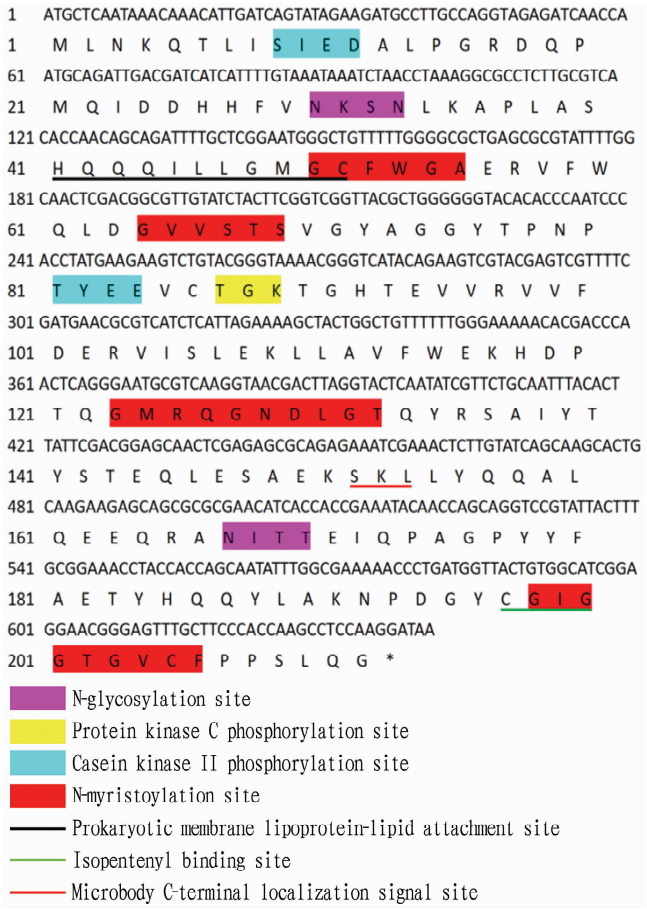
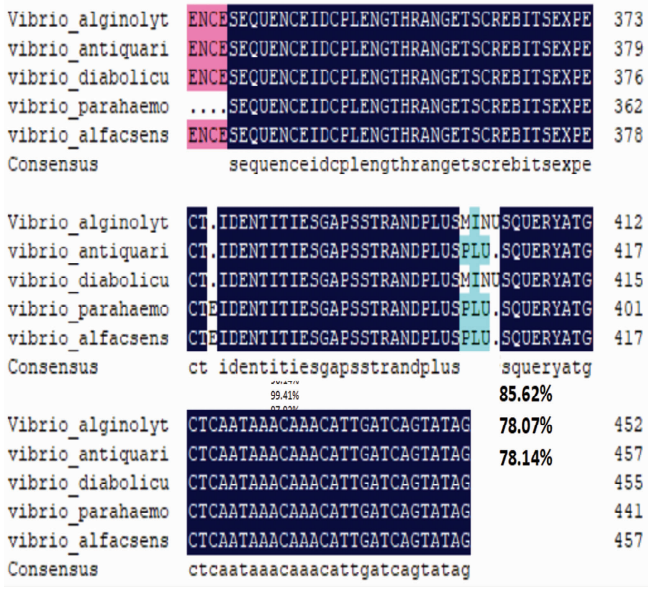


Fig. 2 ***msrA*** **gene nucleotides and its encoded amino acid sequence**



V. alginolyticus (CP072782.1); *V. antiquarius* (CP001805.1); *Vibrio diabolicus* (CP014094.1); *Vibrio parahaemolyticus* (CP078729.1); *Vibrio alfacsensis* (CP032093.1).

Fig. 3 Homology comparison of deduced amino acid sequence of MsrA gene

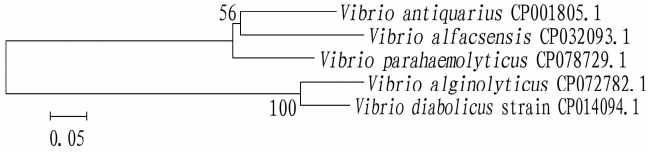


Fig. 4 Phylogenetic tree of MsrA amino acid sequence based on NJ method

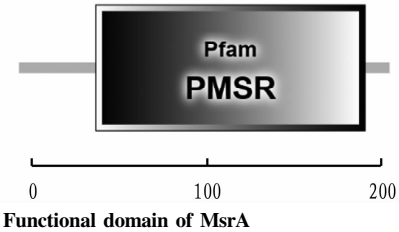


Fig. 5 Functional domain of MsrA

Functional domain, secondary and tertiary structure prediction of MsrA

It was found from the prediction analysis of SMART website that MsrA had a PMSR functional domain (44-198 aa) (Fig. 5).

The results of secondary structure prediction showed that random coils accounted for 46.70%; alpha helixes accounted for 25.47%; extended strands accounted for 20.28%; and beta turns accounted for 7.55% (Fig. 6).

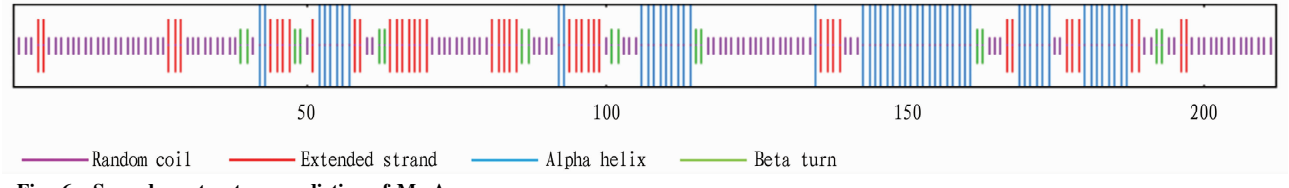


Fig. 6 Secondary structure prediction of MsrA

The amino acid sequence of MsrA was submitted to the SWISS-MODEL program, and homologous proteins were automatically searched as templates (template: Q87SW6.1.A). The single subunit tertiary structure model of MsrA was obtained, and the similarity was 89.15% (Fig. 7).

Protein network interaction

In the protein interaction, it could be found that the proteins adjacent to MsrA were MsrB, PolA, MsrB-2, BAU10_04780, ANP65084.1, ANP65159.1, DsbE_2 and ANP65504.1. Among them, MsrB and MsrA showed the phenomenon of gene fusion; MsrB and MsrB-2 were consistent with MsrA in distribution; and MsrB, PolA, MsrB-2 and SdhA were coexpressed with MsrB (Fig. 8).

PTM modification site analysis of MsrA

The analysis of PTM modification sites showed that MsrA protein had phosphorylation, glycosylation, ubiquitination, acetylation and methylation modification sites, but no hydroxylation modification sites (Table 2).

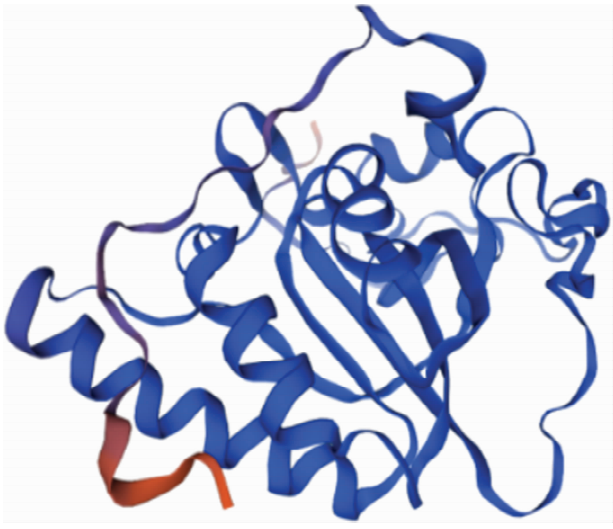


Fig. 7 Tertiary structure prediction of MsrA

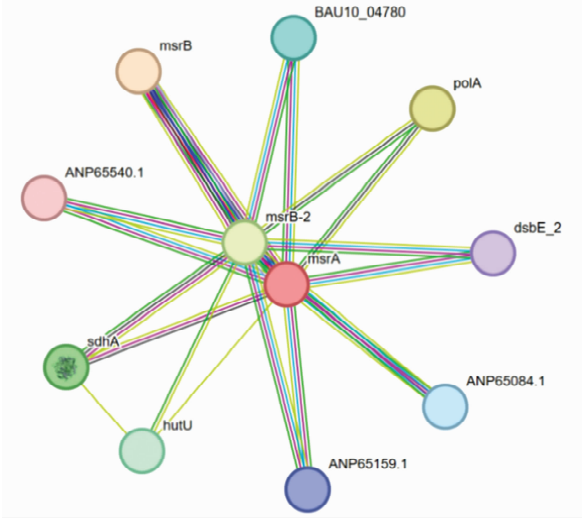


Fig. 8 Protein network interaction of MsrA

Table 2 PTM modification site analysis of MsrA

	Phosphorylation	Glycosylation	Ubiquitination
MsrA	+	+	+
	Acetylation	Methylation	Hydroxylation
MsrA	+	+	−

Discussion and Conclusions

Discussion

Based on the amino acid sequence of *msrA* gene of *V. alginolyticus* HY9901, its physicochemical properties, structure and function, homology, protein network interaction and other parameters were comprehensively compared and analyzed, and the possible role of MsrA protein was reasonably speculated. In the analysis of physical and chemical properties, MsrA protein was stable, but its instability coefficient was as high as 39.32. This result could be reasonably explained in the prediction of protein secondary structure. In the prediction of the secondary structure of MsrA, it was found that the α-helix and β-turn structures accounted for 33.02%, and the random coil structure accounted for 46.70%. It showed that there were many unstable structures in the amino acid secondary structure of MsrA protein, which corresponded to the analysis results of physical and chemical properties, suggesting that we should pay attention to the anti-degradation work of protein when doing related *in-vitro* experiments of MsrA protein.

msrA is a highly conserved gene, which exists in the genomes of many organisms[25-26]. In this study, *msrA* gene (639 bp) was successfully cloned from the genome of *V. alginolyticus* HY9901. The homology analysis of the gene sequence showed that *msrA* of *V. alginolyticus* had high homology, which confirmed the high conservation of *msrA*. On the other hand, some literatures suggest that MsrA protein has a highly conserved characteristic motif GCFWG, and site-directed mutagenesis of each amino acid in this region can lead to a decrease or even complete loss of MsrA activity[27]. The sequence analysis results in this study showed that MsrA of *V. alginolyticus* also had this characteristic motif (50-54 aa), which once again showed that MsrA protein is highly conserved, and it is speculated that it plays an important role in maintaining the basic functions of cells.

The antioxidant repair function of methionine sulfoxide reductase A (MsrA) has been proved in many studies. The study of G St John *et al.*[28] pointed out that MsrA could protect *Escherichia coli* and *Mycobacterium tuberculosis* from oxidative damage of reactive nitrogen intermediates. Mahawar *et al.*[29] found that MsrA could cooperate with chaperonin GroEL to repair the catalase damaged by oxidation, and make *Helicobacter pylori* recover part of its antioxidant function. In this study, MsrA of *V. alginolyticus* had a functional domain PMSR, indicating that it had the function of repairing ROS damaged protein. Therefore, we speculate that MsrA may also participate in the antioxidant stress process of *V. alginolyticus* by repairing oxidative damage proteins.

In addition to anti-oxidative stress, MsrA also plays an important role in influencing bacterial virulence and antibiotic tolerance. Romsang *et al.*[13] tested the virulence of *Drosophila* model and confirmed that the deletion of *msrA* would weaken the virulence of *Pseudomonas aeruginosa*. Singh *et al.*[30] found that *Staphylococcus aureus* treated with cell wall active antibiotics could significantly induce MsrA, and pointed out that bacterial MsrA protein played a role in antibiotic stress response. It suggests that MsrA of *V. alginolyticus* may also be involved in the regulation of its virulence and antibiotic tolerance, which is worthy of further research and discussion. In this study, although no results could directly prove that MsrA is directly related to the virulence or antibiotic resistance of *V. alginolyticus*, MsrA was adjacent to pilus assembly protein (ANP65540.1) in the prediction results of protein network interaction. Pili are a kind of bacterial adhesin, which plays an important role in bacterial virulence and antibiotic tolerance[31]. Therefore, the inference that MsrA may be involved in regulating the virulence and antibiotic tolerance of *V. alginolyticus* is reasonable. In addition, it was also found in the protein network interaction prediction that MsrA and MsrB were adjacent proteins, and there was a relationship of gene fusion and co-expression. Such phenomenon has also been found in many bacteria such as *S. aureus* and *Neisseria gonorrhoeae*[32-33]. It suggests that

MsrA and MsrB may participate in some biological processes or functional pathways together.

Conclusions

The full-length *msrA* gene of *V. alginolyticus* HY9901 is 639 bp, encoding 212 amino acids, and its predicted molecular weight is 23 729.60 Da. The protein has no signal peptide and transmembrane region, and the subcellular localization prediction results show that MsrA is most likely located in the cytoplasm. The amino acid sequence of MsrA contain multiple signal binding sites and has a PMSR functional domain, and its secondary structure is mainly composed of random coils. *msrA* of *V. alginolyticus* is conservative and share high homology with other *Vibrio* species. In protein network interaction, MsrA is adjacent to pilus assembly protein (ANP65540.1), and they may interact to participate in the regulation of virulence and antibiotic tolerance of *V. alginolyticus*. MsrA protein has many PTM modification sites such as phosphorylation and glycosylation sites. The results of this study will lay a foundation for the virulence analysis of *V. alginolyticus* and the study of antibiotic tolerance mechanism.

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(Continued on page 10)

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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