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

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Abstract [**Objectives**] To develop a pair of specific primers for the PCR amplification of the full-length *relA* gene from *Vibrio alginolyticus* strain HY9901, as well as to conduct bioinformatics analysis. [**Methods**] The *relA* gene was amplified through PCR, and the resulting gene sequence was subsequently analyzed using bioinformatics tools, including amino acid sequence prediction, functional site analysis, subcellular localization prediction, and homology comparison. [**Results**] The *relA* gene had a total length of 2 220 bp and encoded 739 amino acid residues. The molecular weight was approximately 84.126 1 kDa, and its isoelectric point was 5.95. The protein lacked a signal peptide and transmembrane regions, while exhibiting multiple phosphorylation sites. Predictions regarding its subcellular localization suggested that it was predominantly situated in the cytoplasm. The amino acid sequence demonstrated a homology of 97% to 99% with other species within the genus *Vibrio*, and it clustered within the same subfamily as *V. antiquarius* and *V. diabolicus*. In the prediction of secondary structure, the proportions of α-helix, extended strand, random coil, and β-sheet were 54.13%, 12.04%, 28.15% and 5.68%, respectively. The similarity between the tertiary structure model and template 5kpw.1.w was 66%. [**Conclusions**] In this study, the *relA* gene of *V. alginolyticus* strain HY9901 has been successfully amplified and analyzed. The structural characteristics and potential functions of the encoded protein have been elucidated, thereby providing foundational data for understanding the role of this gene in *V. alginolyticus*.

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

0 Introduction

Vibrio alginolyticus, a member of the genus *Vibrio* within the family Vibrionaceae, is a gram-negative, anaerobic brevibacterium characterized by its motility, which is facilitated by polar flagella that enable movement in liquid media, as well as lateral flagella that allow for movement on solid media. Research indicates that the optimal culture conditions for *V. alginolyticus* are a pH of 7.62, a temperature of 34.55 ℃, and a salinity of 3.22%[1]. This bacterium is more commonly found in water temperatures ranging from 25 to 32 ℃, suggesting that its pathogenicity is most pronounced in summer. Additionally, a decline in the immune function of the host organism, along with environmental degradation, may further exacerbate the prevalence of this pathogen. The bacterium has the potential to induce diseases in various marine organisms, including snapper, grouper, shrimp, prawn, and shellfish[2]. Additionally, *V. alginolyticus* is recognized as a prevalent pathogen in both humans and aquatic animals[3-4], capable of causing conditions such as otitis media, otitis externa, conjunctivitis, and septicemia in humans. Consequently, *V. alginolyticus* is a substantial threat to both aquaculture and human health. Investigating its pathogenic mechanisms in aquatic animals offers considerable economic and social benefits.

RelA is an enzyme present in bacteria that encompasses the SYNTH, TGS, and ACT structural domains, primarily functioning within the cytoplasm. The high conservation of RelA across bacterial species suggests that this protein plays a crucial and potentially irreplaceable role in cellular life processes. It is responsible for the synthesis of a signaling molecule called (p) ppGpp[5-7]. This molecule is of significant importance in bacterial cells, particularly in their response to environmental stress. (p) ppGpp is a signaling molecule involved in the stringent response, which enables bacteria to detect environmental stresses through its interaction with ribosomes. In the presence of conditions such as nutrient deficiency, DNA damage, or other environmental challenges, bacteria activate an adaptive mechanism known as the stringent response which is mediated by the interaction of RelA with ribosomal tRNA in bacterial cells[8]. During this process, the RelA enzyme is activated and initiates the synthesis of (p) ppGpp. The synthesized (p) ppGpp subsequently binds to the ribosome, resulting in alterations to its translational activity. This modification leads the cell to either slow down or halt the synthesis of specific non-essential proteins, thereby prioritizing the production of proteins that are critical for stress response and survival. In this manner, bacteria are capable of surviving and proliferating in adverse environ-

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mental conditions, thereby rendering RelA a significant translational factor[9]. Additionally, RelA functions as a specific sensor for amino acid starvation, exhibiting robust (p) ppGpp synthesis activity. It is recognized as the most extensively studied long-structural-domain RelA-SpoT homologous enzyme (RSH). When bacteria are subjected to environmental stresses, such as amino acid starvation, the aminoacyl-tRNA binds to the vacant A site of the ribosome, resulting in the formation of a 'starved' ribosome. This condition subsequently activates the (p) ppGpp synthesizing activity of RelA[10]. At this juncture, RelA catalyzes the conversion of ATP and GTP or GDP into AMP and pppGpp or ppGpp, respectively[11].

Currently, the regulatory mechanisms governing the enzymatic activity of RelA remain poorly understood. The specifics of its interactions with ribosomes, as well as the conformational changes it undergoes upon binding to ribosomes, have yet to be elucidated. Consequently, the precise molecular mechanisms underlying the RelA-mediated stringent response are not fully comprehended. In this study, bioinformatic methods were employed to systematically analyze the physicochemical properties, functional sites, phosphorylation sites, protein-protein interaction (PPI) networks, and three-dimensional structural features of the RelA protein. Additionally, amino acid sequence-based homology and phylogenetic analyses of the RelA protein were conducted to establish a foundation for a comprehensive examination of the biological functions of the RelA protein and the elucidation of the functional mechanisms of RelA in the stress response of pathogenic bacteria, in order to offer insights and a scientific basis for the identification and application of novel antibacterial agents that target RelA.

1 Materials and methods

1.1 Materials

1.1.1 Strains. A highly virulent strain of *V. alginolyticus*, designated as HY9901, was isolated and preserved from the bodies of diseased *Lutjanus sanguineus* in the Zhanjiang sea area of Guangdong Province, China, by the laboratory of the Fisheries College at Guangdong Ocean University[12].

1.1.2 Main reagents. Ex*Taq* DNA polymerase was supplied by Takara, while the bacterial genomic DNA extraction kit and DNA gel recovery kit were obtained from Tiangen. The remaining reagents utilized in this study were either imported or domestically produced and were of an analytically pure quality. The synthesis of PCR primers and the determination of their sequences were conducted by Shanghai Sangon Biotechnology Services Co., Ltd. The concentration of the antibiotic used was ampicillin (Ap) at 100 μg/mL.

1.2 Methods

1.2.1 Extraction of total DNA from *V. alginolyticus* HY9901. *V. alginolyticus* strain HY9901 was spread on a TSA plate, from which single colonies were selected and subsequently inoculated onto a TSB medium containing 5% NaCl. The cultures were incubated under shaking conditions at 28 ℃ for over 12 h. An appropriate volume of the bacterial suspension was then transferred to an EP centrifuge tube and centrifuged at 10 000 rpm for 1 min to collect the biomass. The genomic DNA was extracted following the manufacturer's instructions provided with the extraction kit and was stored at −20 ℃ for future use.

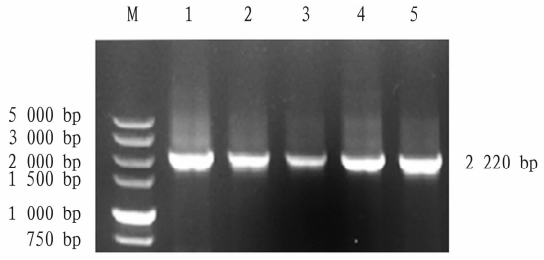
1.2.2 Cloning of the *relA* gene. A pair of primers was designed based on the sequence of the *relA* gene from *V. alginolyticus*. The upstream primer, designated as P1, was ATGGTTGCGGTAAGAAGCGCGCATT, while the downstream primer, designated as P2, was TTAGCCGAGGCGTTTCACCGTCATC. A polymerase chain reaction (PCR) was conducted using the total DNA extracted from *V. alginolyticus* HY9901 as the template. The reaction was conducted under the following conditions: pre-denaturation at 94 ℃ for 5 min; denaturation at 94 ℃ for 30 sec, annealing at 70 ℃ for 30 sec, and extension at 72 ℃ for 40 sec, 35 cycles; and extension at 72 ℃ for 10 min. PCR products were analyzed through electrophoresis on a 1% agarose gel, followed by excision and recovery utilizing a DNA gel recovery kit. These recovered fragments were then cloned into the pMD18-T vector, designated as pMD-*relA*.

1.2.3 Bioinformatics analysis of the *relA* gene from *V. alginolyticus* HY9901. In accordance with the method established by Pang Huanying *et al.*[13], the *relA* gene was translated utilizing DNAMAN software. Signal peptide sequences were predicted through the online analysis tool SignalP 4.0 Server (http://www.cbs.dtu.dk/services/SignalP). The TMHMM Server 2.0 (http://www.cbs.dtu.dk/services/TMHMM) was employed to predict transmembrane structural domains. The distribution of functional sites within the amino acid sequences was analyzed using SoftBerry-Psite (http://linux1.softberry.com/berry.phtml?topic=psite&group=programs&subgroup=proloc). Sequence homology comparison and similarity analysis were conducted via the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A phylogenetic tree was constructed using MEGA 5.0 software. The prediction of functional domains and secondary structure domains of RelA was performed using SMART and SOPMA. The SWISS-MODEL program was utilized for modeling the tertiary structure domains. Additionally, the STRING database (http://string.embl.de/) was employed to investigate PPI networks involving RelA, while Musitedeep was used for the analysis of PTM sites of RelA.

2 Results and analysis

2.1 Cloning of the *relA* gene A specific band of approximately 2 220 bp was successfully amplified by PCR (Fig. 1), which encoded 739 amino acids.

2.2 Physicochemical properties of RelA The RelA protein of *V. alginolyticus* was analyzed utilizing ExPASy software. The analysis revealed that the total number of protein atoms was 11 846, with a molecular structural formula of $C_{3\,712}H_{5\,934}N_{1\,056}O_{1\,115}S_{39}$.

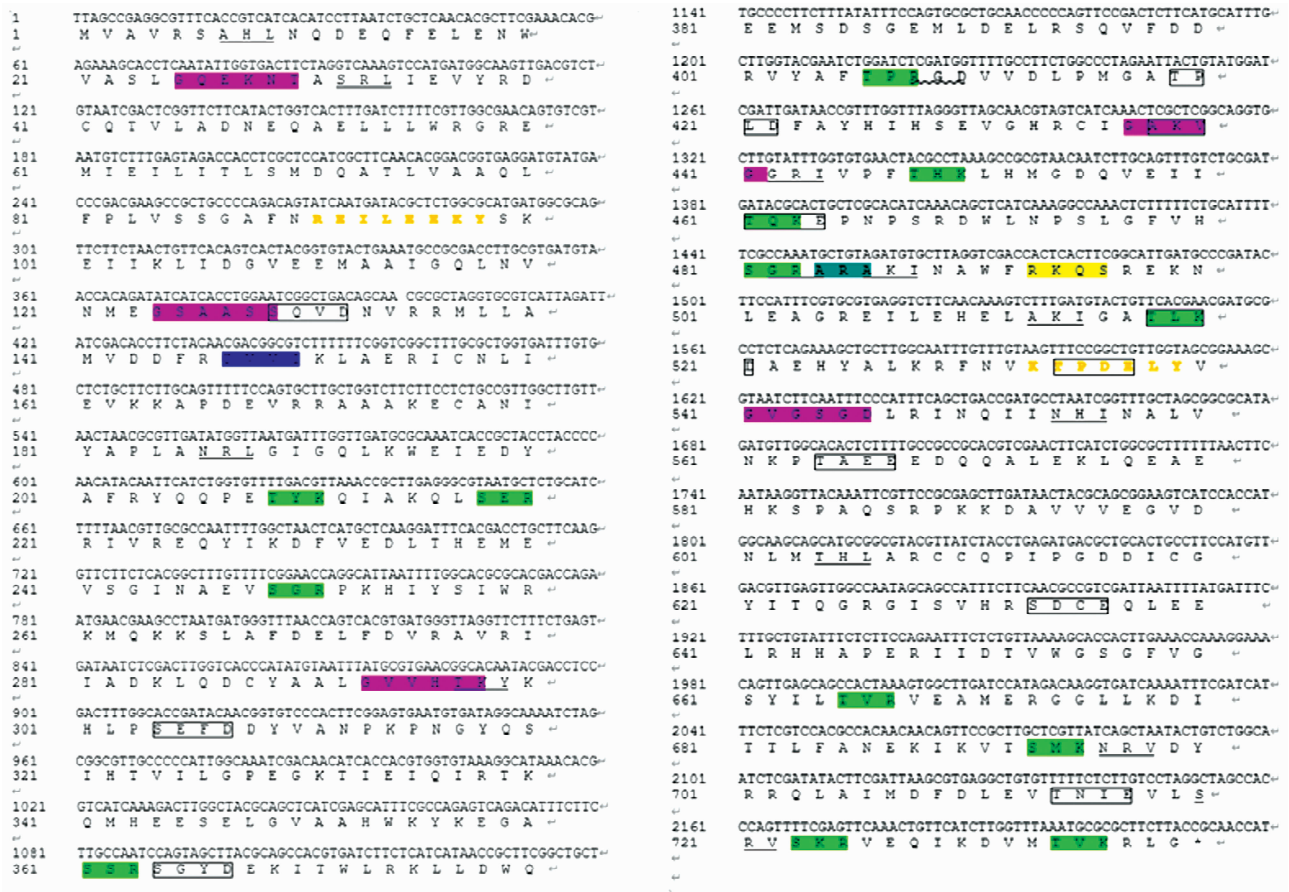


NOTE M. DL5 000 DNA Marker; Lanes 1 – 5. PCR products of the *relA* gene.

Fig. 1 Amplification of the *relA* gene

The theoretical molecular weight was calculated to be 84.126 1 kDa, and the theoretical isoelectric point (pI) was 5.95. The instability coefficient of 45.09 indicated that the protein is of an unstable type. Furthermore, the fat coefficient of 91.96, in conjunction with a total average hydrophilicity of – 0.406, suggested a predominant hydrophobic nature of the protein. The total count of acidic amino acid residues (Asp + Glu) amounted to 113, while the total number of basic amino acids (Arg + Lys) was 14. Additionally, the N-terminal of the protein sequence was identified as methionine (Met). The estimated half-lives for expression of this protein were 20 h in yeast, 10 h in *Escherichia coli*, and approximately 30 h in mammalian reticulocytes when expressed *in vitro*.

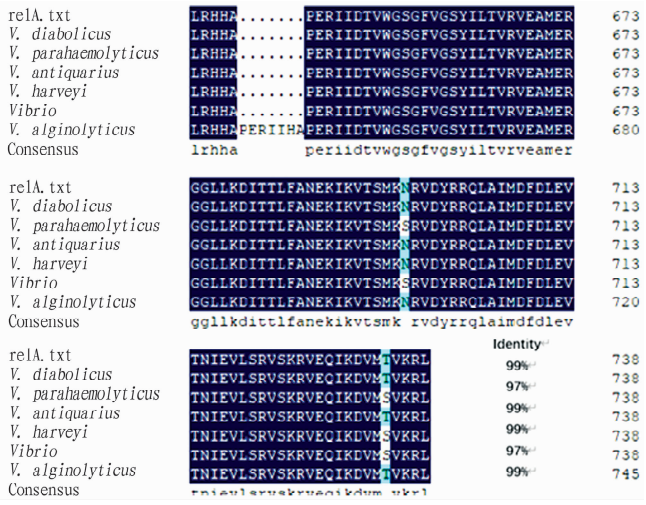
2.3 Sequence analysis of the *relA* gene The prediction of the N-terminal signal peptide structure of the RelA amino acid sequence, utilizing the SignalP 4.0 Server program, indicated the absence of a discernible signal peptide cleavage site within RelA, suggesting that no signal peptide is present. The protein was determined to lack a transmembrane region, as predicted by the TMHMM Server 2.0 program. The predictions generated by the SoftBerry-Psite program indicated that the amino acid sequence encompassed a cAMP- and cGMP-dependent protein kinase phosphorylation site, 13 phosphorylation sites for protein kinase C, 10 phosphorylation sites for casein kinase II, 2 phosphorylation sites for tyrosine kinase, 5 N-terminal myristoylation sites, a Prenyl radical binding site, 14 microsomal C-terminal target signaling sites, and a cell attachment sequence site (Fig. 2). The predicted subcellular localization of the protein indicated that RelA was most likely to be found in the cytoplasm (69.6%), followed by the nucleus (21.7%). The protein was least likely to be located in peroxisomes and mitochondria, with both organelles showing a probability of 4.3%.



NOTE Terminators are denoted by *; yellow highlights indicate cAMP- and cGMP-dependent protein kinase phosphorylation site; green highlights denote phosphorylation sites for protein kinase C; boxed sections represent phosphorylation sites for casein kinase II; gold lettering in bold signifies phosphorylation sites for tyrosine kinases; pink highlights indicate N-terminal myristoylation sites; blue highlights represent Prenyl radical binding sites; underlined text and cyan highlights denotes microsomal C-terminal target signaling sites; and wavy lines illustrate cell attachment sequence sites.

Fig. 2 Nucleotides of the *relA* gene and the corresponding amino acid sequences they encode

2.4 Homology and evolutionary distribution of RelA BLAST analysis demonstrated that the RelA protein of *V. alginolyticus* exhibited a high degree of homology with the RelA proteins of other *Vibrio* species. Specifically, the homology with the amino acid sequences of RelA from *V. antiquarius*, *V. diabolicus*, and *V. harveyi* reached as high as 99%. Furthermore, multiple sequence similarity comparisons indicated that RelA was highly conserved across *Vibrio* species (Fig. 3).



NOTE The strains utilized for comparative analysis include *Vibrio diabolicus* (WP_256956387), *V. parahaemolyticus* (MBE4142554), *V. antiquarius* (WP_301607790), *V. harveyi* (WP_130545004), *Vibrio* sp. (WP_005490307), and *V. alginolyticus* (WP_213869447).

Fig. 3 Homology comparison of amino acid sequences derived from *relA* genes

Utilizing MEGA 5.0 software and the Neighbor-Joining method, the deduced amino acid sequences of RelA were employed to construct a phylogenetic tree in conjunction with other species. The results indicated that the RelA protein of *V. lysogeneticus* HY9901 clustered significantly with *V. antiquarius* and *V. diabolicus* within the same subfamily, suggesting a close phylogenetic relationship among these species (Fig. 4). This finding aligns with the outcomes of traditional classification based on morphological and biochemical characteristics.

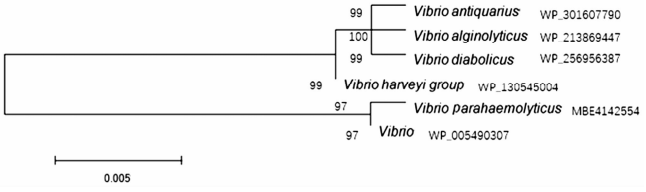
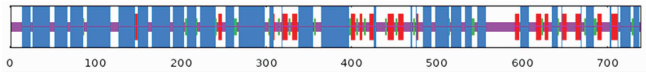


Fig. 4 Phylogenetic tree of RelA amino acids constructed based on the NJ method

2.5 Functional domains, secondary and tertiary structure predictions for RelA The predictions generated by the SMART program indicated the presence of a RelA functional domain (3.85e-62) (Fig. 5). The secondary structure analysis revealed a composition of 54.13% α-helix, 12.04% extended strand, and 28.15% random coil (Fig. 6).

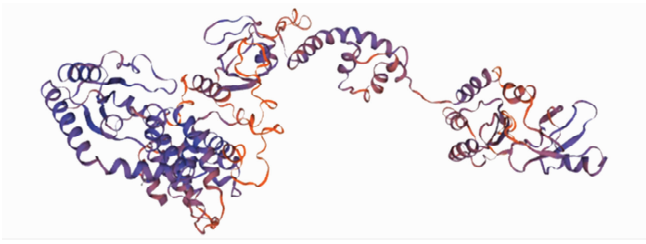
Fig. 5 Functional domains of RelA



NOTE Blue: α-helix; Purple: random coil; Red: extended strand; Green: β-sheet.

Fig. 6 Secondary structure prediction of RelA

The amino acid sequence of RelA was submitted to the SWISS-MODEL program, which conducted an automated search for homologous proteins to identify suitable templates for constructing a tertiary structural model of the RelA single subunit (Fig. 7).



NOTE Template: 5kpw.1.w; Similarity: 66%.

Fig. 7 Prediction of the tertiary structure of RelA

2.6 PPI network of RelA Among the PPI networks, it can be observed that the proteins adjacent to the RelA protein included ANP63797.1, gppA, ndk, ribBA, mazG, sopT, cyaA, ribBA, pykF, and ANP67209.1 (Fig. 8).

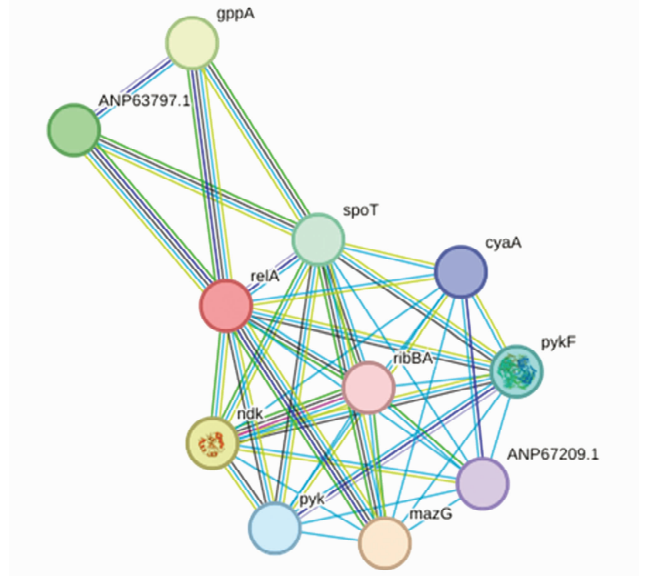


Fig. 8 PPI network of RelA

2.7 Prediction analysis of PTM sites The analysis results of PTM sites indicated that the RelA protein exhibited phosphorylation, glycosylation, ubiquitination, acetylation, and methylation modification sites, while no hydroxylation modification sites were identified.

3 Discussion

V. alginolyticus is a pathogenic bacterium that is prevalent in both humans and aquatic animals[3-4]. It is a substantial threat to both aquaculture and human health. Therefore, investigating its pathogenic mechanisms in aquatic animals is of considerable economic and social benefits. The management of *V. alginolyticus* infections typically involves the use of antibiotics and various chemical agents. However, this approach has resulted in significant concerns regarding drug residues and the emergence of bacterial resistance. In light of these challenges, the identification of effective protective antigens and the development of innovative vaccines have increasingly become focal points of research.

At this stage, bioinformatics analysis serves as the primary method for predicting and analyzing the function and structure of RelA proteins. This approach enables the prediction of the physicochemical properties of proteins, as well as their higher-order structures[14]. Xu Benjin *et al.*[9] developed a prokaryotic expression vector for the *relA* gene, which encodes the stringent response protein in *E. coli*. They employed bioinformatics methods to analyze the structure and function of the RelA protein, thereby establishing a foundation for elucidating the molecular mechanisms underlying the bacterial stringent response mediated by RelA. Yu Chunbo *et al.*[15] investigated the impact of the stringent response-associated (p) ppGpp synthase gene (*relA*) on the heterogeneous resistance of *Acinetobacter baumannii* to polymyxin. Their findings suggest that the *relA* gene, which is involved in the bacterial stringent response, may play a significant role in the development of heterogeneous resistance of *A. baumannii* to polymyxin.

In this study, the *relA* gene of *V. alginolyticus* was successfully cloned. A comprehensive and comparative analysis was conducted on its physicochemical properties, structure and function, homology, and PPI network. Additionally, plausible roles of the RelA protein were hypothesized based on the findings. In the analysis of physicochemical properties, the instability coefficient of the RelA protein was found to be as high as 45.09, indicating that it is an unstable protein. This finding can be elucidated through the prediction of the protein's secondary structure. The prediction of the secondary structure of the RelA protein revealed that α-helix and β-sheet comprised 59.81% of the structure, while extended strand and random coil constituted 40.19%. This finding suggests a predominance of unstable conformations within the amino acid secondary structure of the RelA protein, which aligns with the outcomes of the physicochemical property analysis. This indicates that, when conducting *in vitro* experiments involving RelA proteins, it is imperative to exercise additional caution to prevent protein degradation, thereby ensuring the accuracy and reliability of the experimental results.

RelA is a highly conserved protein. Studies conducted by Xu Benjin *et al.*[9] have demonstrated that RelA exhibits a high degree of conservation among bacterial species. In the present study, a homology analysis of the *relA* gene cloned from the genome of *V. lysogeneticus* HY9901 revealed that the *relA* gene of *V. lysogeneticus* shared significant homology with various *Vibrio* species, thereby supporting the notion of the highly conserved nature of RelA.

RelA protein is extensively distributed across various organisms and plays a crucial role in the synthesis of the signaling molecule (p) ppGpp, which is known to regulate bacterial biofilm synthesis[16]. When the *relA* gene is knocked out, the synthesis level of (p) ppGpp decreases, subsequently inhibiting bacterial growth, motility, and biofilm formation[17]. It has been demonstrated that both elevated and reduced levels of (p) ppGpp significantly impede the growth of *E. coli*. Specifically, elevated levels of (p) ppGpp restrict ribosome synthesis, whereas reduced levels of (p) ppGpp constrain the expression of metabolic proteins[18]. Numerous studies have demonstrated that RelA proteins are subject to various post-translational modifications, including phosphorylation[19], acetylation[20], ubiquitination[21], and methylation[22]. Specifically, both lysine and arginine methylation are present, and these methylation modifications play a crucial role in regulating diverse processes, including the transcriptional activity of NF-κB. In this study, the structure of the N-terminal signal peptide was predicted based on the amino acid sequence of RelA. The results indicated the absence of a distinct signal peptide cleavage site, suggesting that no signal peptide is present in the RelA protein. Consequently, it can be inferred that the RelA protein does not belong to the category of secretory proteins. The predictions generated by the TMHMM Server 2.0 indicate that the protein lacks a transmembrane region. An analysis of the phosphorylation sites within the amino acid sequence of the RelA protein identified a total of 47 phosphorylation sites. This indicates that the spatial distribution and extent of ubiquitination capable of regulating RelA proteins is numerous. The analysis of PTM sites on the RelA protein indicates the presence of multiple modification sites. This observation further suggestes that the RelA protein exhibits significant synthetic activity towards the (p) ppGpp molecule.

An important finding of this study is that RelA proteins, in addition to their significant synthetic activity concerning the stringent response signaling molecule (p) ppGpp, also play regulatory roles in the environmental stress response and in phenotypes associated with virulence. In the research conducted by Yin Wenliang[23], it has been demonstrated that the genes *relA* and *spoT* play a significant role in the response to environmental stress and the regulation of virulence. Although this study does not provide direct evidence linking *relA* to the virulence or environmental stress response of *V. alginolyticus*, the results from the PPI network predictions indicate that the RelA and *SpoT* proteins are adjacent to one another. This proximity suggests that *relA* and *spoT* may collaboratively participate in various biological processes or functional pathways, including the regulation of environmental stress responses and virulence-related phenotypes, *etc.*

A comprehensive investigation into the biological function of the RelA protein and its mechanism of action in pathogenic bacteria responding to stress will yield valuable insights and a scientific foundation for the development of novel antibacterial agents targeting RelA. Furthermore, this research will offer new perspectives for theoretical studies on microbe-environment interactions. In this study, the characterization of the RelA protein was thoroughly examined utilizing bioinformatics analysis tools. This approach estab-

lishes a robust foundation for comprehending the function of the RelA protein in pathogenic bacteria and its involvement in stress response signaling. Furthermore, it offers theoretical support and an experimental basis for the development of novel antibacterial agents targeting the RelA protein.

4 Conclusions

The complete sequence of the *relA* gene was successfully amplified from *V. alginolyticus* strain HY9901. This gene was 2 220 bp in length and encoded 739 amino acid residues. The RelA protein had a relative molecular weight of approximately 84.126 1 kDa, and its isoelectric point was 5.95. Predictive analysis indicated that the RelA protein did not possess a transmembrane region and was predominantly localized in the cytoplasm. Furthermore, the protein exhibited multiple phosphorylation and modification sites, demonstrating 97% to 99% homology with other species within the genus *Vibrio*. The predicted secondary structure was primarily composed of α-helix and random coil, while the tertiary structure exhibited a 66% similarity to the template 5kpw.1.w. The prediction of PPI networks indicated that the RelA protein interacted with adjacent proteins, including ANP63797.1 and gppA. Furthermore, the RelA protein possessed several PTM sites, notably for phosphorylation and glycosylation. By knocking out the *relA* gene in *V. alginolyticus* and performing comparative analysis with the wild-type strain, researchers can further investigate the function and mechanism of action of this gene in transcriptional regulation.

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