Analysis of Transcriptional Differences Between *Vibrio alginolyticus* Strains HY9901 and HY9901Δ*kdpD*

Yashu LIANG[1,2], Weijie ZHANG[1,2], Na WANG[3], Jianyi WEI[4], Huanying PANG[1,2]*

1. Fisheries College, Guangdong Ocean University, Zhanjiang 524025, China; 2. Guangdong Provincial Key Laboratory of Aquatic Animal Disease Control and Healthy Culture & Key Laboratory of Control for Diseases of Aquatic Economic Animals of Guangdong Higher Education Institutes, Zhanjiang 524025, China; 3. Chinese Academy of Inspection and Quarantine, Beijing 100176, China; 4. Guangxi Beihai Nanjiang Aquatic Products Co., Ltd., Beihai 536000, China

Abstract [**Objectives**] To compare the differences in transcriptional expression between the wild-type strain HY9901 and the *kdpD*-deleted strain HY9901Δ*kdpD* of *Vibrio alginolyticus*, and to preliminarily clarify the regulatory role of the *kdpD* gene in the transcriptome of *V. alginolyticus*. [**Methods**] Transcriptome sequencing technology was employed to analyze differences in gene expression between the two strains. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed on the differentially expressed genes. Using 16S rRNA as an internal reference, the expression patterns of selected differentially expressed genes were validated through real-time quantitative PCR. [**Results**] A total of 753 and 1 331 differentially expressed genes were identified, of which 293 were upregulated and 460 were downregulated. A total of 378 functional items were successfully annotated for the Δ*kdpD* mutant, and GO enrichment analysis revealed that these differentially expressed genes were primarily associated with amino acid transmembrane transport and transport activity. KEGG enrichment analysis indicated a predominant involvement in the phosphotransferase system (PTS). Quantitative PCR (qPCR) validation demonstrated that, compared to the wild-type strain, the genes *tolC*, *aspA*, and *bfr* were significantly upregulated in the Δ*kdpD* mutant strain, whereas *glpF* was significantly downregulated, consistent with the transcriptomic data. [**Conclusions**] The deletion of the *kdpD* gene (Δ*kdpD*) markedly affects the transcriptional levels of numerous genes in *V. alginolyticus*, thereby demonstrating the global regulatory role of the *kdpD* gene in this organism. This finding provides foundational data for subsequent functional studies of the *tyeA* gene and the development of subunit vaccines.

Key words *Vibrio alginolyticus*, HY9901, Δ*kdpD* mutant strain, Expression differences

0 Introduction

Vibrio alginolyticus is a significant Gram-negative opportunistic pathogen in marine aquaculture in China. It belongs to the genus *Vibrio* within the family Vibrionaceae and is characterized as a halophilic facultative anaerobic short rod-shaped bacterium. This bacterium is widely distributed in nearshore seawater and estuarine environments and is capable of infecting various cultured aquatic animals, including fish, crustaceans, and shellfish. Meantime, *V. alginolyticus* is a zoonotic pathogen. Human exposure to contaminated seawater or consumption of undercooked aquatic products harboring the bacterium can result in wound infections and acute diarrhea. These infections have led to considerable economic losses in the aquaculture industry in recent years. Lee *et al.*[1] showed that the extracellular polymeric substances (ECP) of *V. alginolyticus* can alter the penaeid prawn plasma components and play a significant role in the treatment of prawn diseases. Chen *et al.*[2-3] proposed that serine proteases are among the most critical factors contributing to prawn mortality, and that protease inhibitors, such as phenylmethylsulfonyl fluoride (PMSF), can effectively inhibit these enzymes.

The pathogenic process of this bacterium is influenced by the host's condition, the virulence of the pathogen, and the culture environment. This process involves sequential stages, including adhesion and invasion, proliferation within the host, and the release of virulence factors that disrupt host metabolism. The primary virulence factors are extracellular products comprising multiple components such as lipopolysaccharides, iron carriers, and serine proteases. The synergistic action of these factors ultimately results in multi-tissue damage and host mortality. Biologically, this bacterium is adapted to the nearshore seawater environment of China, with optimal growth conditions at temperatures ranging from 17 to 35 ℃, pH values between 6.0 and 9.0, and NaCl concentrations of 2% -10%. The likelihood of outbreak and prevalence is significantly increased under the stress conditions present in aquaculture environments.

The two-component signal transduction system is a highly conserved mechanism in prokaryotes for detecting environmental stimuli. Among these systems, the KdpD/KdpE pathway plays a critical role in regulating bacterial potassium ion homeostasis. As a histidine kinase, KdpD is capable of sensing three distinct stimuli: potassium ion concentration, osmotic pressure, and ATP con-

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* Corresponding author. Huanying PANG, doctoral degree, professor, research fields: aquatic veterinary medicine.

centration, thereby maintaining intracellular potassium ion balance in bacteria. Recent research has confirmed that, beyond its fundamental physiological functions, this system is directly involved in the regulation of pathogen virulence and serves as a pivotal determinant of the survival capacity of various pathogens within host immune cells. Current research on the virulence of *V. alginolyticus* predominantly centers on the identification of individual virulence factors, while the comprehensive regulatory mechanisms remain inadequately elucidated. Previous studies have shown that the deletion of the *kdpD* gene (*ΔkdpD*) affects various aspects of *V. alginolyticus*, including its growth rate, biofilm formation, and extracellular protease activity[4]. Song Dawei *et al.*[5] performed cloning and bioinformatics analyses on the effector protein HY322 and the *Va1686* gene, which are associated with the type III secretion system of *V. alginolyticus*, to assess the potential of HY322 as a universal antigen for this species. Liang Caifeng *et al.*[6] conducted an experimental investigation of the *vscH* gene, which encodes a YopR core family protein associated with the type III exportin of *V. alginolyticus* HY9901 strain. They predicted that the B-cell antigenic epitopes identified within the *vscH* gene could serve as valuable references for the subsequent artificial synthesis of antibodies against *V. alginolyticus*. Zhou Zejun *et al.*[7] focused on the molecular chaperone escort protein VscO; through gene cloning and bioinformatics analyses, they established a theoretical foundation for understanding the escort mechanism of the type III secretion system in *V. alginolyticus*.

To date, no studies have reported transcriptional differential analyses comparing the *V. alginolyticus* HY9901 strain with its mutant strain HY9901*ΔkdpD*. In this study, the *tyeA* gene was cloned, and comprehensive bioinformatics analyses were performed to examine its sequence, structural features at multiple levels, physicochemical properties, and other relevant aspects. These findings provide a theoretical foundation for subsequent research on the immune defense mechanisms associated with the *tyeA* gene in *V. alginolyticus* HY9901 strain, as well as for the development and investigation of related subunit vaccines.

1 Materials and methods

1.1 Strains *V. alginolyticus* HY9901 was obtained from a virulent strain maintained in the Disease Laboratory of Guangdong Ocean University, located in Zhanjiang City, Guangdong Province. Both the wild-type strain and the *ΔkdpD* monoclonal mutant strain were inoculated into 1 mL of TSB medium and incubated overnight at 28 ℃ with shaking at 180 rpm. Subsequently, these cultures were diluted 1 ∶ 100 into fresh TSB medium and further incubated for 12 h before being stored for future use.

1.2 Main reagents The primary reagents utilized included TSB medium and Rx*Taq* DNA polymerase, which was obtained from Takara Company. The bacterial genomic DNA extraction kit and DNA gel recovery kit were procured from All Style Gold Company. Standard molecular weight DNA markers were sourced from TaKaRa. All other reagents were either imported or of domestic analytical grade.

1.3 Instruments PCR instruments (Bio-Rad and TaKaRa Companies); constant voltage and constant current electrophoresis apparatus (Beijing Liuyi Electrophoresis Apparatus Factory); benchtop high-speed low-temperature centrifuge (Eppendorf Company); CHA-S electric heating constant temperature incubator (Shanghai Boxun Industrial Co., Ltd. Medical Equipment Factory); clean bench (Shanghai Huilong Instrument Co., Ltd.).

1.4 Database construction and sequencing

1.4.1 RNA extraction and detection. The bacterial suspension was collected by centrifugation and subsequently dissolved in Trizol reagent (Takara Bio, Inc.) for RNA extraction. Thereafter, rigorous quality control was performed on the RNA samples. The primary quality assessment method employed was the Agilent 2100 Bioanalyzer, which enabled precise evaluation of RNA integrity and quantification of total RNA.

1.4.2 Library construction and quality inspection. Following the successful quality assessment of the extracted RNA, messenger RNA (mRNA) was isolated by removing ribosomal RNA (rRNA) from the total RNA. Subsequently, a fragmentation buffer was added to randomly cleave the mRNA into short fragments. Using the fragmented mRNA as a template and random oligonucleotides as primers, the first strand of complementary DNA (cDNA) was synthesized employing the M-MuLV reverse transcriptase system. Thereafter, the RNA strand was degraded by RNase H. The second cDNA strand was then synthesized using DNA polymerase I, with dNTPs in which dTTP was replaced by dUTP, resulting in a second strand containing A/U/C/G. The purified cDNA underwent end repair, followed by the addition of an A tail, and subsequent ligation with a sequencing adapter. cDNA fragments approximately 370 – 420 bp in length were then selected using AMPure XP magnetic beads. The second strand of the cDNA containing U was degraded using the USER enzyme prior to PCR amplification. The resulting PCR products were purified again with AMPure XP magnetic beads to obtain the final library. Initial quantification of the constructed library was performed using a Qubit 2.0 fluorometer. The library was diluted to a concentration of 1.5 ng/μL, and the insert size was assessed using an Agilent 2100 bioanalyzer. Upon confirmation that the insert size met the expected criteria, the effective concentration of the library (exceeding 2 nM) was precisely quantified by qRT-PCR to ensure library quality.

1.4.3 Sequencing run. Following the successful qualification of the library inspection, libraries were selected based on effective concentration and target offline data volume, after which Illumina sequencing was conducted. Four fluorescently labeled dNTPs, DNA polymerases, and splicing primers were added to the flow cytometry plates to facilitate sequencing amplification. During the extension of complementary strands in each sequencing cluster, the incorporation of each fluorescently labeled dNTP emitted a corresponding fluorescence signal. The sequencer detected these fluo-

rescence signals and, through computer software, converted the optical signals into sequencing peaks, thereby generating the sequence information of the target fragments.

1.5 Information analysis The image data of sequencing fragments generated by the high-throughput sequencer were converted into sequence data (reads) using CASAVA base calling software. The resulting files were in FASTQ format. Following the filtering of raw data, quality assessment was conducted by evaluating the sequencing error rate and examining the GC content distribution, yielding clean reads for subsequent analyses. Genomic mapping of the filtered reads was performed using Bowtie2 software (Langmead, B. 2012)[8]. Utilizing gene annotation information from the reference genome, the total mapped reads were assigned to corresponding genomic regions. Based on the alignment positions of genes on the reference genome, the number of reads covering each gene, including newly predicted genes, was quantified from start to end.

1.6 Differential analysis Following the quantification of gene expression, it is essential to perform statistical analyses on the expression data to identify genes exhibiting significantly different expression levels across various conditions. In experiments involving more than two groups, cluster analysis can be applied to different gene sets to group genes with similar expression patterns. Such genes may share common functions or participate in related metabolic and signaling pathways.

1.7 Enrichment analysis This study employed the clusterProfiler[9] software to perform GO functional enrichment analysis and KEGG pathway enrichment analysis on the differential gene set. Specifically, the differential gene set comprised genes identified through differential expression analysis and subsequently annotated using the GO or KEGG databases. The background gene set included all genes subjected to differential analysis and annotated within the GO or KEGG databases. Enrichment analyses were conducted on all differential gene sets, including both up-regulated and down-regulated gene sets, for each differential comparison combination.

1.8 Verification of transcriptome results The wild-type strain HY9901 and the Δ*kdpD* strain were cultured in TSB at 28 ℃ for 12 h. Eight differentially expressed genes were randomly selected from the transcriptome data of the Δ*kdpD* mutant strain. The primers used are listed in Table 1. Following the method described by Li *et al.*, RNA was extracted and cDNA was synthesized. Quantitative real-time PCR was then performed to validate the transcriptome data obtained from the mutant strain.

1.9 Analysis of differential gene expression in mutant strains Four differentially expressed genes associated with *V. alginolyticus*, specifically *tolC*, *glpF*, *aspA*, and *bfr*, were identified from the transcriptomic data of the Δ*kdpD* mutant strain. The primers used are listed in Table 1. The expression levels of these differentially expressed genes in the mutant strains were analyzed by real-time PCR, as described in Section **2.8**.

Table 1 qPCR primers

Primer	Sequence	Source
q-ydhR-F	CAAGAGCCGGGCATGATTTG	CP017918.1
q-ydhR-R	CTCGTACTTCGACTACGCCC	
q-dhaL-F	TGGTAAAGCAGAGCTGGGTG	CP072782.1
q-dhaL-R	ATTTCAATCGTGGCAACCGC	
q-mcp-F	TCACAAGAGGGCAAGAGCAG	MT007791.1
q-mcp-R	GGCAGCATTGGAGGCAATTT	
q-flaG-F	CCACTCGAAATGCGACTCCT	AF069392.3
q-flaG-R	ATGGCTGAAAGCCGTCAAGA	
q-luxR-F	CGGTGTCGGTGAGTTCTCTT	MH121058.1
q-luxR-R	CACCCTTATCCAGATGGCCG	
q-paaf-F	TACTGAGCGACAACGGACTG	ACZB01000002.1
q-paaf-R	TCAGCCGTGCTAACGTTGAT	
q-yscW-F	CGACGGCTACTTTCCACCTT	CP060386.1
q-yscW-R	CAGGCAACTGAACACCTTGG	
q-lcrV-R	CGACAGGAGCAACAGACA	MG907044.1
q-lcrV-F	CGTTTGTCCCGAGATAGTT	
q- tolC-F	CAAGATCTTCGCTTGCTGCC	EU625278.1
q- tolC-R	CCACCTACCAACGCTCTCTC	
q-glpF-F	TGTTCGCTTACTTCGCTGGT	UHIJ01000001.1
q-glpF-R	TGTGGAAGGTAAGCGGCAAT	
q-aspA-F	GCATGGTACTCACGTAGCGG	DQ097160.1
q-aspA-R	CTTTCACAAGACCAGAAGAGTAACC	
q-bfr-F	TCAAGCCAAGTTGCGTCTCT	CP013484.1
q-bfr-R	ATGCGATTGCCTACGCTGAA	
16S-F	TTGCGAGAGTGAGCGAATCC	NR_044825.2
16S-R	ATGGTGTGACGGGCGGTGTG	

2 Results and analysis

2.1 Differential analysis The volcano plot effectively illustrates the distribution of differentially expressed genes across each comparative group (Fig. 1). Transcriptome sequencing analysis of the HY9901 strain and the Δ*kdpD* mutant strain identified 753 and 1 331 differentially expressed genes, respectively. Compared to the HY9901 strain, the Δ*kdpD* strain exhibited upregulation of 293 genes and downregulation of 460 genes.

2.2 Enrichment analysis

2.2.1 GO enrichment. The Gene Ontology (GO) database provides a comprehensive framework for describing gene functions, categorized into biological processes, cellular components, and molecular functions. A significance threshold of $p < 0.05$ was applied for GO functional enrichment analysis. From the enrichment results, the 30 most significant terms were selected and visualized using a histogram (Fig. 2). Among the 378 differentially expressed genes annotated with GO functions related to the Δ*kdpD* strain, enrichment in amino acid transmembrane transport and transport activity was particularly notable.

2.2.2 KEGG enrichment. The Kyoto Encyclopedia of Genes and Genomes (KEGG) is a comprehensive database that integrates genomic, chemical, and systemic functional information. A threshold of adjusted *p*-value (padj) less than 0.05 was applied to identify significantly enriched KEGG pathways. Based on the KEGG

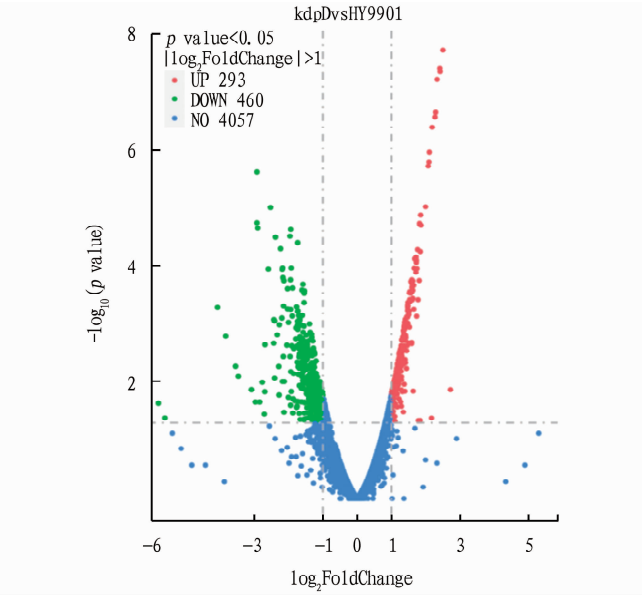


Fig. 1 Differential analysis results

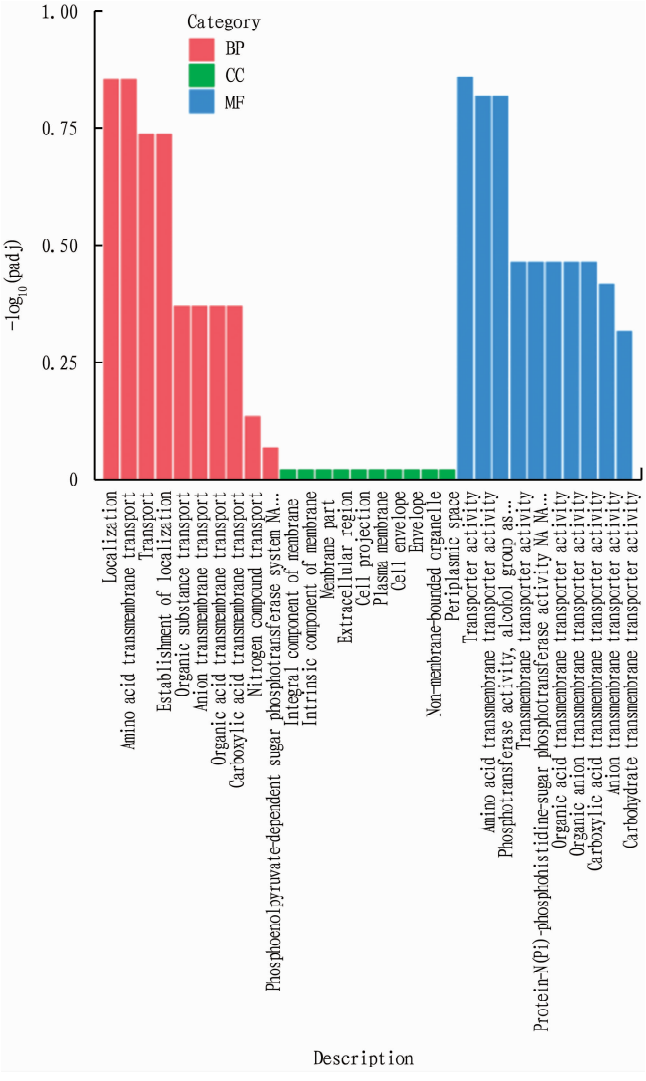


Fig. 2 GO enrichment

enrichment analysis, the top 20 most significant pathways were selected and visualized using scatter plots, as illustrated in Fig. 3. In the figure, the horizontal axis represents the ratio of differentially expressed genes annotated to a given KEGG pathway relative to the total number of differentially expressed genes, while the vertical axis denotes the KEGG pathways. The size of each dot corresponds to the number of genes annotated within the respective pathway, and the color gradient from red to purple indicates the degree of enrichment. Notably, the differentially expressed genes in the *ΔkdpD* mutant strain were predominantly enriched in the phosphotransferase system (PTS).

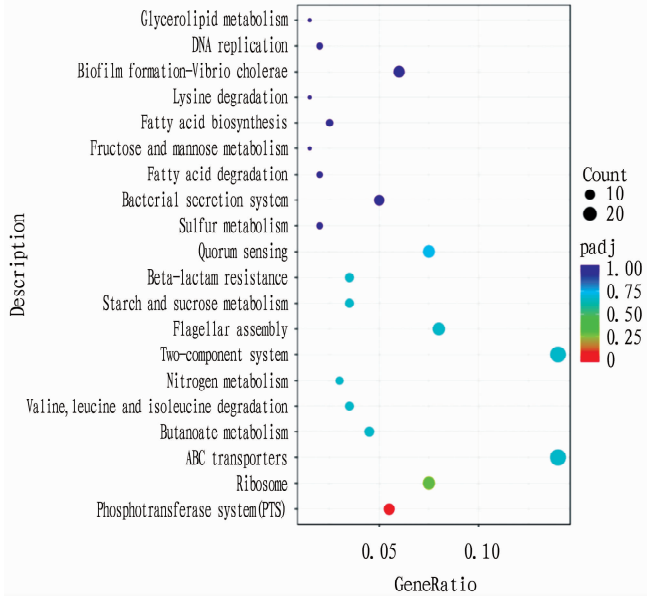


Fig. 3 KEGG enrichment

2.3 Verification of selected differentially expressed genes using qPCR The qPCR results showed that the expression pattern of the mutant *ΔkdpD* mRNA was consistent with the RNA-Seq data, indicating that the transcriptome data were accurate and the analysis results were reliable (Fig. 4).

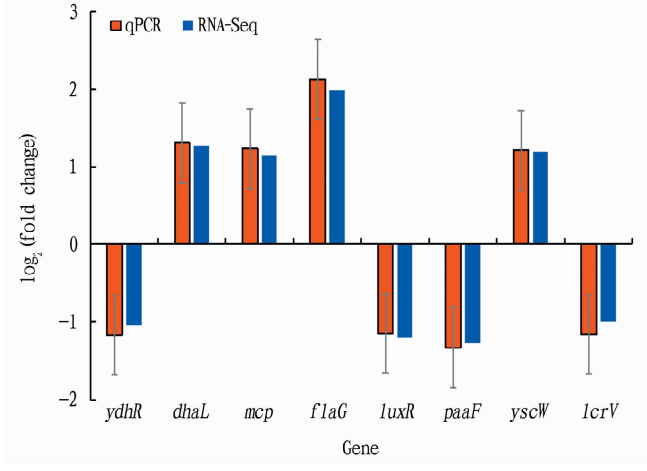


Fig. 4 Verification of selected differentially expressed genes using qPCR

2.4 Analysis of differential gene expression in mutant strains Using 16S rRNA as the internal reference, fluorescence quantitative PCR analysis demonstrated that, compared to the wild-type strain, the Δ*kdpD* mutant strain exhibited significant upregulation of *tolC*, *aspA*, and *bfr*, alongside downregulation of *glpF* (Fig. 5).

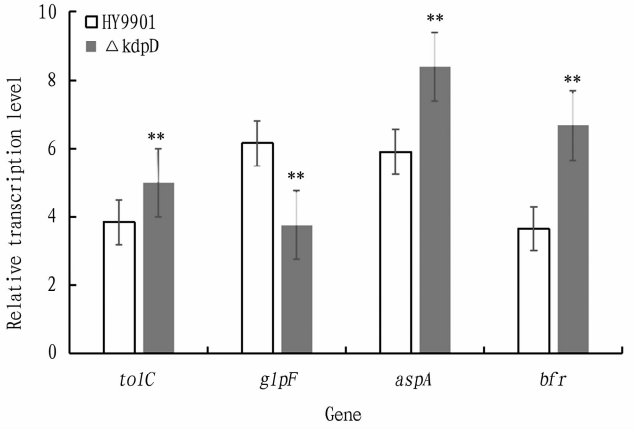


Fig. 5 Analysis of differential gene expression in mutant strains

3 Discussion

The KdpD/KdpE two-component system plays a critical role in the intracellular survival and pathogenicity of various pathogenic bacteria. In *Staphylococcus aureus*, upregulation of *kdpD/kdpE* transcription enhances bacterial survival within macrophages, whereas the absence of this system diminishes bacterial survival capacity[10]. The *kdpD/kdpE* mutant strain of *Yersinia pestis* is more readily eliminated in the human neutrophil environment[11]. The *kdpD* gene in *Salmonella typhimurium* is essential for nematode infection and intracellular survival within macrophages; mutant strains exhibit reduced pathogenicity toward nematodes, along with significantly impaired intracellular proliferation and cytotoxicity[12]. These observations are generally attributed to the bacteria's ability to acquire potassium in the potassium-deficient environment of phagocytic vesicles. Nevertheless, certain experimental findings contradict the potassium ion-dependent model. For instance, in a human blood environment with adequate potassium ion concentrations, *S. aureus* continues to exhibit upregulated expression of the *kdpD/kdpE* genes, and deletion of these genes results in diminished bacterial survival. Beyond phagocytosis, the transcriptional activity of this system is also induced by biofilm formation and exposure to neutrophil bactericidal extracts, conditions that do not necessarily involve potassium limitation. It is important to emphasize that bacterial physiological activity is influenced by the phosphorylation status of the KdpD/KdpE proteins rather than solely by gene transcription levels. Some perspectives challenge the primary role of the Kdp-ATPase in potassium transport within *S. aureus*, but this conclusion is derived from studies involving single-copy system knockouts. Given that some strains possess two sets of KdpD/KdpE systems, further investigation is required to verify this claim.

Existing research has confirmed that KdpE can directly bind to the promoters of virulence genes. The knockout of the *kdpD/kdpE* genes alters the transcription of hundreds of genes, including those encoding surface proteins, capsule synthesis, toxins, metalloproteinases, hemolysins, and numerous other virulence-associated genes. However, the evolutionary mechanisms underlying the regulation of virulence genes by KdpD/KdpE remain unclear. It is hypothesized that virulence gene promoters have evolved KdpE binding sites or that virulence islands regulated by this system have been acquired through horizontal gene transfer. Regulatory proteins originally associated with non-pathogenic functions have been co-opted by pathogens to participate in the regulation of pathogenicity. Previous studies have predominantly focused on the role of the Kdp system in potassium homeostasis, often overlooking its involvement in toxicity regulation. Therefore, comprehensive multi-species investigations employing gene knockout-complementation, transcriptome, and *in vivo* infection models are warranted to elucidate the regulatory network of this system under conditions of potassium limitation, oxidative stress, osmotic stress, and host infection. Such studies will enhance the understanding of the functions of the Kdp system in non-intracellular parasites and closely related strains exhibiting diverse lifestyles.

This study employed transcriptome sequencing to compare the gene expression profiles of the wild-type strain HY9901 and the Δ*kdpD* mutant strain of *V. alginolyticus*. Differential gene annotation and cluster analyses were performed, followed by the planned construction of deletion and complementary strains via gene recombination technology to assess growth, stress resistance, cellular infection, and *in vivo* virulence phenotypes. Transcriptome analysis identified a total of 753 differentially expressed genes in the Δ*kdpD* mutant strain, of which 293 were upregulated and 460 were downregulated. GO functional enrichment analysis revealed significant involvement in amino acid transmembrane transport and transport activity, while KEGG pathway analysis indicated enrichment primarily in the PTS. qPCR validation demonstrated that, relative to the wild-type strain, *tolC*, *aspA*, and *bfr* were significantly upregulated in the Δ*kdpD* mutant strain, whereas *glpF* was significantly downregulated. This study provides an initial comprehensive overview of the global transcriptomic alterations induced by *kdpD* deletion, thereby providing foundational data for subsequent functional analyses of the *tyeA* gene and the development of vaccines.

4 Conclusions

This study investigated the transcriptional expression differences between the wild-type strain HY9901 and the Δ*kdpD* mutant strain of *V. alginolyticus* using transcriptome sequencing. A total of 753 differentially expressed genes were identified, of which 293 were upregulated and 460 were downregulated. These differentially expressed genes were predominantly enriched in pathways related to amino acid transmembrane transport, transport activity, and the PTS. qPCR validation demonstrated that the expression levels of

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process. Building upon a solid foundation of core professional competencies, it broadens students' career development paths, aiming to cultivate composite talents in Chinese medicine who possess both professional expertise and practical innovation capabilities. The five-pronged reform framework encompassing curriculum, pedagogy, practice platforms, faculty, and evaluation constructed in this study, refined through pilot teaching practice, can be promoted to acupuncture and tuina programs nationwide in universities of Chinese medicine. Subsequent efforts require sustained deepening of university-industry collaboration to further integrate the chains of teaching, practice, and achievement transformation. Continuously improve the integrated talent cultivation model to support the high-quality development of the Chinese medicine health industry.

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tolC, *aspA*, and *bfr* were significantly upregulated, whereas *glpF* expression was significantly downregulated. These findings confirm that deletion of the *kdpD* gene substantially alters the transcriptional profile of numerous genes in *V. alginolyticus*. This study provides an initial elucidation of the regulatory role of the *kdpD* gene on the transcriptome of *V. alginolyticus*, thereby establishing a theoretical basis for future investigations into the biological functions of the *tyeA* gene and the development of subunit vaccines.

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