Cloning, Bioinformatics and Transcriptional Expression Analysis of the *exsB* Gene in *Vibrio alginolyticus*

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Abstract [**Objectives**] Using the virulent strain HY9901 of *Vibrio alginolyticus* as the research object, the differential expression of the *exsB* gene under stress from different culture media was investigated, and the function of this gene was preliminarily inferred. [**Methods**] First, the *exsB* gene was cloned by PCR and subjected to bioinformatics analysis. Then, the expression of this gene was analyzed by real-time fluorescence quantitative PCR. [**Results**] The open reading frame (ORF) of the *exsB* gene is 657 bp in length, encoding 218 amino acids. The ExsB protein has no significant signal peptide cleavage site, and no transmembrane region was found. The amino acid sequence contains multiple active sites, such as N-terminal glycosylation sites. Phylogenetic tree analysis showed that ExsB of *V. alginolyticus* is relatively closely related to the corresponding protein of *Vibrio* sp. JCM 19053. ExsB has one main functional domain: QueC (queuosine biosynthesis protein), which catalyzes the ATP-dependent conversion of 7-carboxy-7-deazaguanine (CDG) to 7-cyano-7-deazaguanine ($preQ_0$). The secondary structure of ExsB is predominantly α-helices. The expression level of *exsB* was highest in bile salt TSB medium, followed by DMEM medium, and the value in each of the two media was significantly higher than that in ordinary TSB medium. [**Conclusions**] Bile salt environments and DMEM medium can induce the positive transcription of ExsB, a regulatory protein of the T3SS in *V. alginolyticus*.

Key words *Vibrio alginolyticus*; *exsB* gene; Bioinformatics; Transcriptional expression
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Vibrio alginolyticus is a common pathogen of cultured animals, causing fin tissue ulceration and hepatopancreatic enlargement in fish, as well as white spot and hemorrhage in shrimp[1]. In severe cases, it leads to large-scale mortality and substantial economic losses[2]. It is also one of the 12 human pathogenic species of the genus *Vibrio*, with relatively strong infectivity[3]. Similar to most Gram-negative bacteria, the pathogenicity of *V. alginolyticus* depends on its interactions with the host, and the type III secretion system (T3SS) is an important part of this process. Studies have shown that mutations in T3SS regulatory genes can weaken bacterial motility and reduce pathogenicity to zebrafish, demonstrating that the T3SS is an important virulence system[4]. T3SS regulatory proteins mainly regulate gene expression and protein secretion.

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There have been studies demonstrating that ExsB is a transcriptional regulator of T3SS-related apparatus proteins and plays a stabilizing role in the outer membrane ring of the T3SS[5]. Some scholars[6] investigated the three regulatory proteins of T3SS1 in *Vibrio parahaemolyticus* and found that T3SS1 expression depends on the interaction among ExsA, ExsC, and ExsD: ExsD binds to ExsA and inhibits T3SS1 expression, whereas ExsC binds to ExsD and releases ExsA, thereby promoting its expression. However, the function of the T3SS regulatory protein ExsB in *V. alginolyticus* remains unclear. Therefore, the *exsB* gene of *V. alginolyticus* HY9901 was cloned, and based on bioinformatics analysis, the transcriptional expression levels of *exsB* under different culture conditions were detected and compared to infer the effects of the intestinal and cellular environments on its expression, so as to preliminarily characterize ExsB and provide a theoretical basis for subsequent functional studies.

Materials and Methods

Materials

Strains and vectors The virulent strain of *V. alginolyticus*, HY9901, was isolated from crimson snapper (*Lutjanus erythropterus*) infected with vibriosis in the coastal waters of Zhanjiang Port. It is preserved in Guangdong Provincial Key Lab of Pathogenic Biology and Epidemiology for Aquatic Economic Animals[7].

Main reagents The DNA gel extraction kit was purchased from Tiangen Biochemical Technology (Beijing) Co., Ltd., and rTaq DNA polymerase was purchased from TaKaRa Biotechnology

(Dalian) Co., Ltd. Primer synthesis and DNA sequencing were performed by Guangzhou Biotechnology Engineering Co., Ltd. The TSB liquid medium contained tryptone 15 g, soytone 5 g, and NaCl 20 g, and was made up to 1 000 ml with distilled water. After adjusting the pH to 7.2, the medium was dispensed, autoclaved, and stored at 4 ℃.

Methods

Extraction of total bacterial DNA from *V. alginolyticus* HY9901 *V. alginolyticus* HY9901 was inoculated into TSB liquid medium at a ratio of 1 ∶ 100 and cultured at 28 ℃ and 200 rpm for 18 h. A 1 ml aliquot of the bacterial culture was taken and centrifuged at 5 000 r/min for 5 min. The supernatant was discarded, and the pellet was washed twice with PBS. Next, 200 μl of sterile double-distilled water was added to the pellet, and the mixture was pipetted to homogeneity and boiled at 100 ℃ for 5 min. After centrifugation at 12 000 r/min for 5 min, the supernatant was collected as the bacterial genomic DNA template and stored at −20 ℃ for later use.

Cloning of the *exsB* gene Based on the *V. alginolyticus* genome sequence in GenBank, primers for *exsB* were designed using Primer 5: forward primer F: ATGGCTACAACAAAAGCACTTGTGG, and reverse primer R: CTATTTGCTCGCTAAGTATTCTTCT. Using the total DNA of HY9901 as the template, PCR was performed in a 20 μl reaction system as follows: 95 ℃ for 5 min, 33 cycles (95 ℃ for 30 s, 58 ℃ for 45 s, 72 ℃ for 2.5 min), and 72 ℃ for 10 min. After the PCR reaction was completed, 1.0% agarose gel electrophoresis was performed on 4 μl of the PCR product for verification. The remaining PCR product was purified according to the instructions of the DNA gel extraction kit and sent to Guangzhou Biotechnology Engineering Co., Ltd. for sequencing.

Bioinformatics analysis The sequence was subjected to bioinformatic analysis using various online tools and software[8] (Table 1).

Table 1 Online programs and software used for bioinformatics analysis

Name	Website	Function
NCBI	http://blast.ncbi.nih.gov/Blast.cgi	Sequence homology alignment and similarity analysis
ExPASy Proteomics Server	http://web.expasy.org/protparam/	Prediction of protein physicochemical properties
clustalw	http://www.genome.jp/tools-bin/clustalw	Amino acid homology alignment analysis
SignalP 4.1	Server http://www.cbs.dtu.dk/services/SignalP	Prediction of signal peptides
TMHMM Server 2.0	http://www.cbs.dtu.dk/services/ TMHMM	Prediction of transmembrane domains
SoftBerry-Psite	http://linux1.softberry.com/berry.phtml? Topic = psite&group = programs&subgroup = proloc	Prediction of the distribution of amino acid functional sites
SMART	http://smart.embl-heidelberg.de/	Prediction of functional domains
SOPMA	https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl? page = npsa_sopma.html	Prediction of secondary structure
SWISS-MODEL	http://www.swissmodel.expasy.org	Construction of protein three-dimensional structure
MEGA 7.0	–	Construction of phylogenetic tree

Differential expression of the *exsB* gene in different culture media qRT-PCR (quantitative real-time reverse transcription PCR) was conducted to detect the expression of the *exsB* gene under three culture conditions: TSB containing 0.05% bile salt (simulating the intestinal environment), DMEM (Dulbecco's modified Eagle medium, a high-glucose medium; simulating the intracellular environment), and ordinary TSB. Specific primers were designed based on the conserved sequence of *exsB*. 16S rRNA was used as the reference gene, and SYBR Green I was applied for labeling. The primers are shown in Table 2.

Table 2 Primers used for real-time fluorescence quantitative PCR

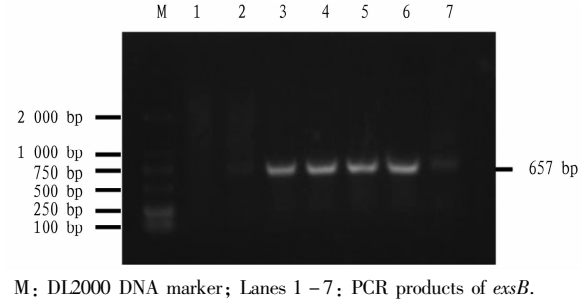
Primer	Sequence (5′-3′)
RT-*exsB*-F	CACAAAGTCCTGGATGTAAAA
RT-*exsB*-R	TCCCGTAATGATGTCTTGAA
RT-16S rRNA-F	TTGCGAGAGTGAGCGAATCC
RT-16S rRNA-R	ATGGTGTGACGGGCGGTGTG

The HY9901 bacterial culture with an OD_{600} of 0.5 was inoculated into the above media at a ratio of 1 ∶ 100 and cultured with shaking at 28 ℃ for 12 h. The bacterial cells were then collected, and total RNA was extracted and reverse-transcribed into cDNA, which was used as the template for qRT-PCR amplification. The specific procedures were performed according to the method described by Peng *et al.*[9].

Results and Analysis

Cloning of the *exsB* gene

A gene-specific band of approximately 700 bp was obtained by PCR amplification (Fig. 1). Sequencing showed that the open reading frame (ORF) of the *exsB* gene is 657 bp in length, encoding 218 amino acids (Fig. 2). The corresponding GenBank accession number is MN385415.



M: DL2000 DNA marker; Lanes 1 – 7: PCR products of *exsB*.

Fig. 1 Cloning of the *exsB* gene

Physicochemical properties of the *exsB* gene

The ExPASy software was employed to analyze the ExsB protein of *V. alginolyticus* HY9901, and the results are shown in Table 3.

Table 3 Physicochemical properties of the ExsB protein

Protein	Molecular mass kDa	Theoretical pI	Instability index
ExsB	24.07	4.93	46.30

Protein	Grand average of hydropathicity (GRAVY)	Number of acidic amino acids (Asp + Glu)	Number of basic amino acids (Arg + Lys)
ExsB	−0.057	26	17

Sequence analysis of the *exsB* gene

The N-terminal signal peptide structure of the ExsB amino acid sequence was predicted using the SignalP 4.1 Server program. The results revealed that the protein has no significant signal peptide cleavage site and is not a secretory protein. Online detection using TMHMM Server 2.0 found no transmembrane region in ExsB.

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1    ATGGCTACAACAAAAGCACTTGTGGTTTTTTCCGGTGGGCAGGACTCTACGACCTGCTTA
1     M  A  T  K  A  L  V  V  F  S  G  G  Q  D  S  T  T  C  L
61   ATCGATGCATTAAATACATATGATGAAGTTCATGCTATAACGTTTGATTATGGCCAGCGT
21    I  D  A  L  N  T  Y  D  E  V  H  A  I  T  F  D  Y  G  Q  R
121  CATTCAGATGAAATAGATATCGCTATCGAATTAGGTAAGCAACTTAACATCAAAAGCCAC
41    H  S  D  E  I  D  I  A  I  E  L  G  K  Q  L  N  I  K  S  H
181  AAAGTCCTGGATGTAAAATCAATTAATGAGTTAGCGATAAGCTCGCTGACAAGAGACAAC
61    K  V  L  D  V  K  S  I  N  E  L  A  I  S  S  L  T  R  D  N
241  ATTCCGGTATCTAATGACTTAAGCGACGCTAACGTACCCAATACATTCGTTCCAGGGAGA
81    I  P  V  S  N  D  L  S  D  A  N  V  P  N  T  F  V  P  G  R
301  AACATTCTCTTTCTTACTTACGCATGTATCCATGCTTACCAGCTTGGCATTCAAGACATC
101   N  I  L  F  L  T  Y  A  C  I  H  A  Y  Q  L  G  I  Q  D  I
361  ATTACGGGAGTTTGCGAAACAGACTTTAGTGGTTACCCTGACTGCCGAGACAACTTTATT
121   I  T  G  V  C  E  T  D  F  S  G  Y  P  D  C  R  D  N  F  I
421  CGCTCTCTAAATCTCTCTTGCAATTTAGGTATGGATAAAGCCTTCAATATTAAGACTCCT
141   R  S  L  N  L  S  C  N  L  G  M  D  K  A  F  N  I  K  T  P
481  TTAATGTGGCTTACTAAAGCCGAGACCTGGGCTCTAGCTGATAAGCATAATATGTTGGAA
161   L  M  W  L  T  K  A  E  T  W  A  L  A  D  K  H  N  M  L  E
541  TTTGTTCAAAACAATACTTTAACATGTTACAACGGAATCGTTGGTAAAGGCTGTGGTGAA
181   F  V  Q  N  N  T  L  T  C  Y  N  G  I  V  G  K  G  C  G  E
601  TGCCCAGCGTGCCTACTGCGACAAAGCGGCTTAGAAGAATACTTAGCGAGCAAATAG
201   C  P  A  C  L  L  R  Q  S  G  L  E  E  Y  L  A  S  K  *
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*: stop codon; Light gray shading: N-terminal glycosylation site; Oval: protein kinase C phosphorylation site; Box: casein kinase II phosphorylation site; Single underline: N-terminal myristoylation site; Double underline: alkenyl binding site; Wavy line: microbody C-terminal targeting signal site.

Fig. 2 Nucleotide sequence of the *exsB* gene and its encoded amino acid sequence

Online prediction using SoftBerry-Psite showed that the amino acid sequence contains: 2 N-terminal glycosylation sites (150-153 aa, 193-196 aa), 1 protein kinase C phosphorylation site (4-6 aa), 3 casein kinase II phosphorylation sites (27-30 aa, 70-73 aa, 171-174 aa), 5 N-terminal myristoylation sites (13-18 aa, 14-19 aa, 39-44 aa, 129-134 aa, 156-161 aa), 2 alkenyl binding sites (20-23 aa, 213-216 aa), and 2 microbody C-terminal targeting signal sites (5-7 aa, 171-173 aa) (Fig. 2).

Evolutionary analysis of ExsB

Multiple alignment of the obtained protein sequences was performed using ClustalX software, and a phylogenetic tree of ExsB was constructed using MEGA 7.0 molecular evolutionary genetics analysis software based on the alignment results. Phylogenetic tree analysis (Fig. 3) showed that ExsB of *V. alginolyticus* clustered with the corresponding protein of *Vibrio* sp. JCM 19053 within the same subclade, indicating that they are relatively closely related in evolutionary terms.

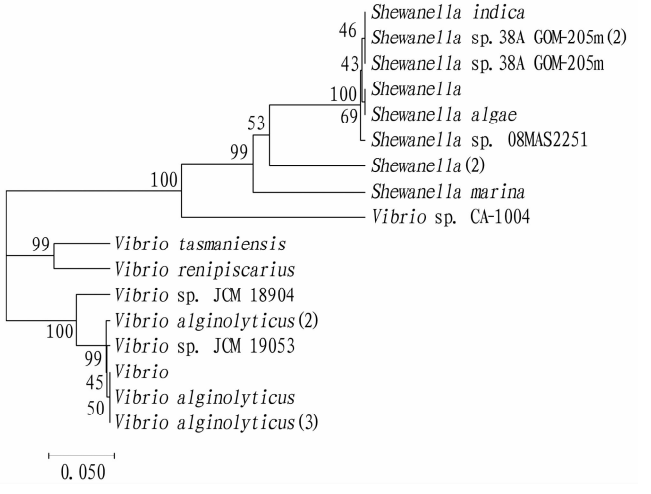


Fig. 3 Phylogenetic tree of ExsB amino acid sequences constructed based on the NJ method (Accession No. MN385415)

Prediction of the functional domains and secondary structure of ExsB

SMART prediction showed that ExsB contains one major functional domain, QueC (Fig. 4). SOPMA prediction indicated that its secondary structure consists of 41.28% alpha helix, 30.28% random coil, 19.27% beta turn and 9.17% extended strand (Fig. 5).

Expression analysis of the *exsB* gene

The experimental results are shown in Fig. 6. The expression levels of the *exsB* gene differed among different culture media. No significant difference was observed between bile salt TSB and DMEM medium, but the expression levels in both were significantly higher than that in ordinary TSB medium. The highest expression level was observed in bile salt TSB medium, and it was approximately six times that in ordinary TSB medium ($P < 0.05$). The expression level of the *exsB* gene in DMEM medium was slightly lower than that in bile salt TSB, but high glucose could promote *exsB* expression, and the expression level was still much higher than that in ordinary TSB medium ($P < 0.05$).

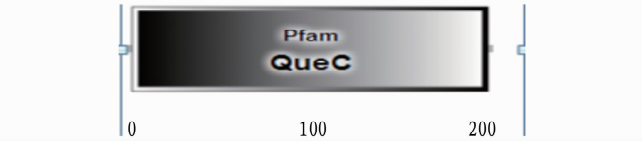
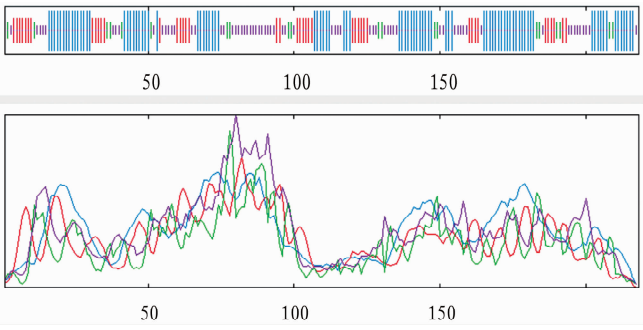
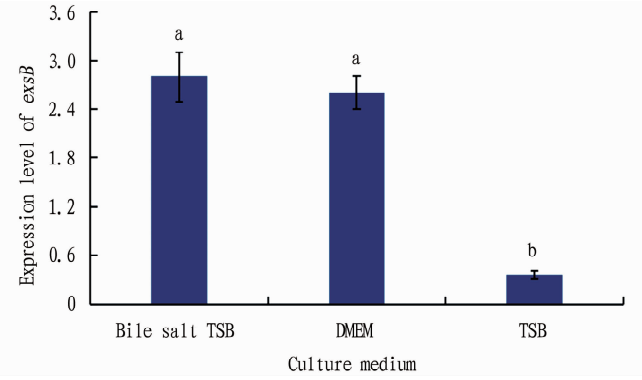


Fig. 4 Predicted domain of ExsB protein



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

Fig. 5 Secondary structure prediction of ExsB protein



Letters following the values in the figure indicate the results of Duncan's multiple range test. Values (mean ± standard error) with the same superscript letter indicate no significant difference, while different letters indicate a significant difference ($P < 0.05$).

Fig. 6 Expression levels of the *exsB* gene in different culture media detected by fluorescence quantitative PCR

Discussion

The type III secretion system (T3SS) is a conserved virulence apparatus in bacteria, which plays an important role in the infection of hosts by *V. alginolyticus*, and is a hotspot in research on *Vibrio* pathogenicity. As a T3SS regulatory protein of *V. alginolyticus*, ExsB is closely related to the pathogenic mechanism. Some external environmental factors, such as pH, temperature and salinity, can regulate the expression of pathogenicity-related proteins in bacteria, thereby affecting their pathogenicity[10]. Therefore, investigating the differential expression of *exsB* in *V. alginolyticus* under different stress factors is of great significance.

In this study, the *exsB* gene of *V. alginolyticus* was selected as the research object. On the basis of gene cloning, bioinformatics analysis was performed on this gene. The results showed that the open reading frame of this gene is 657 bp in length, with a theoretical molecular weight of approximately 24.07 kD, encoding 218 amino acids. Its theoretical isoelectric point is 4.93, indicating an acidic nature. ExsB has an instability index greater than 40, indicating that ExsB is unstable. The T3SS translocates secreted proteins from the cytoplasm into host eukaryotic cells through a needle-like structure spanning the bacterial inner and outer membranes (such as coiled-coil regions and transmembrane segments)[11]. Since ExsB has no transmembrane region or signal peptide, it is presumed to be a soluble protein. Its sequence contains 2 N-terminal glycosylation sites, 1 protein kinase C phosphorylation site, 2 alkenyl binding sites, 3 casein kinase II phosphorylation sites, 5 N-terminal myristoylation sites, and 2 microbody C-terminal targeting signal sites. Secondary structure prediction showed that ExsB is predominantly composed of α-helices and random coils, which is similar to the structures of most T3SS proteins in *V. alginolyticus*.

Protein sequences of various *Vibrio* species were aligned, and a phylogenetic tree constructed using MEGA 7.0 revealed that ExsB of *V. alginolyticus* clustered in the same subclade with the corresponding protein of *Vibrio* sp. JCM 19053. It indicates their close genetic relationship, consistent with biochemical characteristics and morphological classification. SMART analysis identified its main functional domain as QueC (queuosine biosynthesis protein, 7-cyano-7-deazaguanine synthase). In the absence of an external nitrogen source, QueC catalyzes the ATPdependent conversion of 7-carboxy-7-deazaguanine (CDG) to 7-cyano-7-deazaguanine (preQ0)[12]. Queuosine is one of the most complex modifications of tRNA, which is ubiquitously present in eukaryotes, and affects many important biological functions such as toxic effects and cell differentiation. Rahul Gaur *et al.*[13] confirmed through genetic screening in *Escherichia coli* that *queC* (*ybaX*) participates in the first step of queuosine synthesis, and participates in the upstream synthesis of $preQ_0$ using GTP as the starting molecule.

Through realtime fluorescence quantitative PCR analysis, the differential expression of *exsB* in *V. alginolyticus* under different culture media was obtained. The results showed that under bile salt TSB stress, the bacterial cells grew normally, and *exsB* expression was the highest, approximately six times that in ordinary TSB, and the expression in DMEM was slightly lower than that in bile salt TSB, but still much higher than that in ordinary TSB. T3SS gene transcription and expression are influenced by environmental conditions and regulatory proteins. Most reported T3SS regulatory proteins in *Vibrio* have been studied in vitro. For example, in T3SS1 of *Vibrio* parahaemolyticus, ExsA acts as a positive regulator and ExsD as a negative regulator. ExsA directly binds to the upstream regions of VP1668 and VP1687 and is presumed to act on the T3SS promoter sequence[14]. In *Vibrio cholerae*, VttRA and VttRB regulate T3SS structural genes and effector protein secretion under bile salt conditions, with ToxR and VttRA acting upstream of VttRB[15]. These findings are consistent with the present study, suggesting that bile salts affect T3SS expression in *Vibrio*. However, few reports have described the regulation of the

V. alginolyticus T3SS gene cluster by ExsB, and the specific factors that activate T3SS transcription remain to be determined.

As a regulatory protein, ExsB has also been studied in other bacteria. In *Pseudomonas aeruginosa*, knockout of *exsB* reduces virulence. Its deletion leads to decreased in vitro secretion of T3SS proteins and affects the regulatory gene operon. Eukaryotic cell infection and in vitro assays have shown that ExsB facilitates the correct assembly of the type III secretion apparatus on the bacterial cell surface, thereby enabling infection of host cells[16]. In addition, Qu *et al.*[17] investigated the transcriptional regulatory mechanism of ExsB, one of the matrix structural proteins of *Bacillus thuringiensis*. They demonstrated that *exsB* is mainly controlled by the SigmaK factor, and that GerE exerts a direct positive regulatory effect on *exsB* at the transcriptional expression level. At present, there have been no reports on the T3SS regulatory protein ExsB in *V. alginolyticus*.

Predictions of protein physicochemical properties, functional sites and higher-order structure are the basis for functional studies. In this study, bioinformatics analysis of ExsB can reduce blindness in subsequent functional experiments and improve the success rate. As a transcription regulation-related protein, ExsB is presumed to be upregulated under environmental stress and to activate T3SS gene transcription. Under induction by bile salt TSB and DMEM, *exsB* expression was upregulated, which may enhance the ability of *V. alginolyticus* to adapt to the external environment and facilitate its proliferation in the intestinal tract and within cells. These results help to explore the causes of septicemia in fish and shrimp and human diarrhea caused by *V. alginolyticus*, and to reveal the role of ExsB in physiological functions and virulence regulation.

Conclusions

In this study, the *exsB* gene of *V. alginolyticus* HY9901 was successfully cloned and sequenced, and bioinformatics analysis was performed on its encoded protein ExsB. ExsB is a hydrophilic intracellular protein lacking a signal peptide and transmembrane region, possessing a QueC functional domain and multiple potential posttranslational modification sites. Phylogenetic analysis showed that it is relatively closely related to the homologous protein of *Vibrio* sp. JCM 19053. Transcriptional expression analysis indicated that both bile salt TSB and DMEM culture conditions could significantly upregulate *exsB* expression, and the highest expression was observed in bile salt TSB, suggesting that bile salt and highglucose environments may induce ExsB mediated T3SS regulation and environmental adaptation in *V. alginolyticus*. These results lay a foundation for further research on the function of ExsB in the pathogenic mechanism of *V. alginolyticus*.

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