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# A live attenuated strain of HY9901Δ*vscB* provides protection against *Vibrio alginolyticus* in pearl gentian grouper (♀*Epinephelus fuscoguttatus* × ♂*Epinephelus lanceolatu*)

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## ABSTRACT

*Vibrio alginolyticus*, a bacterial pathogen in fish and humans, expresses a type III secretion system (T3SS) that is critical for pathogen virulence and disease development. In this study, the T3SS chaperone protein gene *vscB* was cloned from *V. alginolyticus* wild-type strain HY9901. The results showed that the *vscB* gene has a length of 429 bp and encodes 142 amino acids. The amino acid sequence homology comparison results showed that VscB had the highest similarity with that of *Vibrio parahaemolyticus* VscB, reaching 91%. The mutant strain HY9901Δ*vscB* was constructed by the in-frame deletion method. The HY9901Δ*vscB* mutant showed an attenuated swarming phenotype and a 23-fold decrease in virulence to pearl gentian grouper (♀*Epinephelus fuscoguttatus* × ♂*Epinephelus lanceolatu*). However, the HY9901Δ*vscB* mutant showed no difference in morphology, growth, biofilm formation and extracellular protease (ECPase) activity. The results of antibiotic susceptibility testing showed that the wild-type HY9901 is more sensitive to Doxycycline, Minocyline and Kanamycin than in the HY9901Δ*vscB* mutant. A total of 95 differentially expressed genes were screened by transcriptome sequencing analysis of HY9901 and strain Δ*vscB*, revealing 57 genes up-regulated and 38 genes were down-regulated, respectively. qRT-PCR was employed to analyze the transcription levels of T3SS-related genes showing that Δ*vscB*, had decreased expression of *vopN* and *vscO* and increased expression of *hop*, *vscN*, *vscK*, *vscL* and *vopS* compared to the wild strain. Finally, grouper vaccinated via

intraperitoneal (IP) injection with HY9901Δ*vscB* had a high serum antibody titer with a relative percent survival (RPS) of 77.6% following challenge with the wild-type HY9901. Real-time qPCR assays showed that vaccination with HY9901Δ*vscB* enhanced the expression of immune-related genes, including MHC-I, IgM, and CD8α both in the liver and spleen, indicating that the mutant *V. alginolyticus* strain is able to induce humoral and cell-mediated immune responses in pearl gentian grouper. These results demonstrate that the HY9901Δ*vscB* mutant could be used as an effective live attenuated vaccine to combat *V. alginolyticus* infection in pearl gentian grouper.

**Keywords:** *Vibrio alginolyticus*; T3SS; *vscB*; live attenuated vaccine; pearl gentian grouper

## Introduction

In recent years, with the development of the aquaculture industry, vibriosis outbreaks have become an important disease issue. *Vibrio spp*. pathogenic to aquatic animals have caused huge economic losses to aquaculture (Lafferty et al., 2015; Mahbub et al., 2011; Haldar, 2012) with *Vibrio alginolyticus* representing a common serious threat to the health of cultured fish.

The pearl gentian grouper is a hybrid fish which is cultivated as a cross between female grouper *Epinephelus fuscoguttatus* × male grouper *Epinephelus lanceolatu*. It is a widely cultured marine fish with an important economic value and increasing market demand in China, but development of the industry is restricted by vibriosis outbreaks (Chen et al., 2019; Wei et al., 2020).

*V. alginolyticus* is mainly distributed in marine environments and is an opportunistic pathogenic bacteria of marine organisms (López et al., 2009). Under suitable conditions, *V. alginolyticus* can rapidly reproduce, out-competing other colonizing bacteria becoming the dominant species, which following infection of the mouth or wounds leads to death and large fish losses to industry (Sadok et al., 2013).. At present, the main prevention and means of controlling vibriosis in grouper are through use of chemical drugs and antibiotics (Zhao et al., 2010; Cai et al., 2007), leading to significant increased antimicrobial resistance (AMR) to such drugs (Cai et al., 2007; Chen et al., 2002; Wang et al., 2008). Vaccination provides a means of prophylaxis without damaging the environment and mitigates issues relating to AMR. Thus vaccine development for *V. alginolyticus* has become the focus of much research

for preventing vibriosis in grouper.

The type III secretory system (T3SS) plays an important role in the infection process of *V. alginolyticus* (Bennett and Hughes, 2000; Yao et al., 2012; Galán and Collmer, 1999; Cornelis and Van Gijsegem, 2000). Although there have been many reports on the mechanism of T3SS effector proteins (Wattiau et al., 1994; Wattiau et al., 1996), relatively few studies have elucidated the role of T3SS chaperone proteins, which are required to maintain the effector protein stability in the bacterial cytoplasm (Spaeth et al., 2009). Molecular chaperone proteins also assist the secretion or transport of the T3SS effector proteins (Stebbins and Galán, 2001) and some can also be transported into host cells and participate in host intracellular functions (Birtalan et al., 2002; Day and Plano, 1998). VscB protein is an important component of the T3SS, and is speculated to play an important role in host invasion. By functional domain prediction, VscB is highly similar to Tir chaperone protein, which belongs to the Tir chaperone protein (CesT) family. VscB belongs to chaperone protein (YscB) protein family. YscB functions as a molecular chaperone for NosA protein, which gives *Yersinia pestis* the ability to change host cell morphology (Day et al., 2003). It is reasonable to hypothesise that VscB, as a molecular chaperone in the *V. alginolyticus* T3SS, plays an important role in the regulation of T3SS.

Therefore, in this study, the gene encoding *vscB* of *V. alginolyticus* was cloned and analysed by bioinformatics, providing evidence for the role of VscB in *V. alginolyticus* T3SS. At the same time, the deletion strain of *vscB* was constructed to investigate the molecular chaperone functions of VscB in *V. alginolyticus in vitro* and its role in

virulence. In addition, the *vscB* deletion strain of *V. alginolyticus* was tested as a protective live attenuated vaccine in immunised pearl gentian grouper.

## Materials and methods

### Bacterial strains, fish and culture conditions

The bacterial strains and plasmids used in this work are listed in Table 1. *V. alginolyticus* wild-type strain HY9901 was isolated from *Lutjanus erythopterus* (Cai et al., 2007) and was utilized as the parent strain for constructing the mutant strain Δ*vscB* and the complementation strain C-Δ*vscB*. *V. alginolyticus* was cultured on tryptic soy broth (TSB, Huankai Co Ltd., Guangzhou, China) or on 1.5% TSB agar plates (TSA) at 28℃. *Escherichia coli* strains were cultured in Luria-Bertani (LB, Huankai Co Ltd., Guangzhou, China) or on LB agar at 37 ℃. When required, the appropriate antibiotics were added: ampicillin (Amp, 100μg $mL^{-1}$); kanamycin (Km, 50μg $mL^{-1}$); chloramphenicol (Cm, 25μg $mL^{-1}$).

Pearl gentian grouper (average weight 25.0 ± 2.0g) were obtained from a commercial fish farm in Zhanjiang, China, and kept in seawater in a recirculation system at 20-22℃ for two weeks before the experiment. Fish were considered healthy by sera agglutination and bacteriological recovery tests as described previously (Pang et al., 2018).

Groupers were anaesthetized with tricaine methane sulfonate (MS222) (Kuer, Anwei, China) before injections and sample collection. Animal experiments complied with ethical standards and were approved by Guangdong Provincial Key

Laboratory of Pathogenic Biology and Epidemiology for Aquatic Economic Animals Ethics Committee.

**Cloning and sequencing of the *vscB* gene from *V. alginolyticus* HY9901**

A pair of primers *vscB*-F1 and *vscB*-R1 were designed as shown in Table 2 according to the *V. alginolyticus* gene sequence (GenBank Number: GU074526). PCR was performed in a Thermocycler (Bio-Rad, CA, USA) under the following optimized amplification conditions: an initial denaturation at 95℃ for 5 min, followed by 30 cycles of 95 ℃ for 30 s, 60 ℃ for 30 s and 68 ℃ for 30 s. 5 μ L of each amplicon was examined on 1% agarose gels, stained with ethidium bromide. The PCR product was recovered from the agarose gel to ligate into the pMD18-T vector and transformed into *E. coli* DH5α (Table 1). The inserted fragment was sequenced by Sangon Biological Engineering Technology Services Co., Ltd. (Shanghai, China). Similarity analyses of the determined nucleotide sequences and deduced amino acid sequences were performed by BLAST program (http://blast.ncbi.nlm.nih.gov/ Blast.cgi) and aligned using the program Clustal-X (version 1.81). Protein analysis was conducted with ExPASy tools (http://expasy.org/tools/). Location of the domain was predicted using the InterProScan program (http://www.ebi.ac.uk/Tools /pfa/iprscan/).

**Construction of a Δ*vscB* and complementation of the mutant**

The Δ*vscB* mutant was constructed with allelic exchange mutagenesis. Firstly, two specific pairs of primers were designed to obtain the *vscB* upstream homologous arm fragment A (Primers: *vscB*-MF1 and *vscB*-MF2) and downstream homologous arm fragment B (Primers: *vscB*-MR1 and *vscB*-MR2). Then, fragment A and B were used

as templates, and the AB fragments were fused together by overlapping PCR. The AB fusion fragment was connected to the suicide vector pLP12, and the recombinant product was transformed into *E.coli DH5α*. The positive clones were selected and the recombinant plasmid PLP12-*vscB* was extracted. PLP12-*vscB* was transformed into *E. coli β2163*. The positive clones were selected and used for the conjugation with *V. alginolyticus*, and after undertaking homologous recombination twice, the Δ*vscB* mutant was successfully constructed with PCR identification (Primers: *vscB*-TF and *vscB*-TR).

The *vscB* fragment (Primers: *vscB*-RF and *vscB*-RR) and pBAD33cm-rp4 vector fragment (Primers: pBAD30-ZF and pBAD30-ZR) were amplified, connected and then transformed into *E.coli DH5α.* The positive clones were selected (Primers: pBAD30-mcf-TF and pBAD30-mcf-TR) and sequenced to confirm the successful construction of *vscB* complementation strain (C-*vscB*).

**Characterization of the Δ*vscB***

The wild-type HY9901 strain and Δ*vscB* mutant was respectively cultured in TSA at 28°C for 24 h. Cell morphology was then investigated by transmission electron microscopy (TEM).

*Genetic stability of mutants HY9901ΔvscB*

HY9901Δ*vscB* was inoculated onto a TSA plate and passed blindly for 30 generations. Its genetic stability was determined by PCR. Growth ability was measured as previously described (Zhou et al., 2013). The wild-type HY9901 strain, HY9901Δ*vscB* and C-*vscB* were cultured in TSB for 24 h at 28 °C. The HY9901,

HY9901Δ*vscB* and C-*vscB* were inoculated in TSB at the ratio of 1: 100 ($OD_{600}$ = 0.5), determination of $OD_{600}$ was performed every 2 h, and repeated three times per group.

*Swarming motility*

Swarming motility was assayed using the method of Young et al (Young et al., 1999) and initiated by spotting 2 mL of an overnight culture at the center of agar plates containing 0.6% agar. The swarming diameter was measured after 24 h incubation. All of experiments were performed in triplicate.

*Extracellular protease activity*

Extracellular protease (ECPase) activity was performed according to the method of Windle and Kelleher (Windle and Kelleher, 1997). The wild-type strain HY9901, HY9901ΔvscB and C-vscB were each innoculated onto a TSA plate coated with aseptic cellophane, cultured at 28℃ for 24 h, washed with sterile PBS, centrifuged at 4 ℃ for 30 min, and the supernatant was filtered to obtain extracellular products. Inactivated sample (supernatant was boiled for 10 min) was used as a blank control.

*Crystal violet ammonium oxalate staining*

With reference to the method (Kierek and Watnick, 2003), wild strains HY9901, HY9901Δ*vscB* and C-*vscB* ($OD_{600}$ = 0.5) were transferred to a 96-well plate (200 μL/well, 6 replicate wells, negative control TSB) and the plates were incubated at 28 ℃. Samples were taken at 12 h, 24 h, 48 h and 72 h, methanol fixed for 20 min, then stained with Crystal violet ammonium oxalate dye for 15 min. Finally, 95 % alcohol was then added and incubated at room temperature for 30 min. $OD_{570}$ was determined by using a Multimode Plate Reader.

*Confocal Laser Scanning Microscopy (LSCM)*

The wild strain HY9901 and the mutant strain HY9901Δ*vscB* were diluted 50-fold, added to a glass bottom culture dish(spec: type 28.2mm, class diameter 20mm) (Wuxi NEST, Wuxi, China), and statically cultured in a 37 °C biochemical incubator for 24 h, gently washed three times with physiological saline, and 10 % SYTO9 green added. Incubation of bacteria with fluorescent dyes was carried out in the dark for 20 min, bacteria were washed three times with saline, mounted in 40 % saline-glycerol and observed by confocal microscopy. The excitation wavelength was 488 nm, scanned from the bottom to the top of the biofilm, with Z-sections obtained 1 μ m apart. Biofilm parameters - biomass and maximum thickness were determined.

*$LD_{50}$ determination*

The injection concentrations of wild-type strain HY9901Δ*vscB* and C-*vscB* were $10^4$, $10^5$, $10^6$, $10^7$ and $10^8$ cfu / mL. In the experimental group, 100 μL bacterial solution was injected into each tail by intramuscular injection. The control group was injected with 100 μL of PBS in the same manner. Mortalities of experimental fish were recorded over a 14 day period until the mortality rate was stable. The morbidity induced by *V. alginolyticus* was determined, and the $LD_{50}$ of mutant and wild strain was calculated by Koch method (Reed and Muench, 1938) with three replicates per group.

**Antibiotic susceptibility**

The susceptibility patterns of the HY9901,Δ*vscB* and C-*vscB* to 30 different antibiotics were determined according to the disc diffusion method using TSA (Bauer et al., 1966), and the diameters of the inhibition zones were measured using Vernier

calipers. Resistant, intermediate and susceptible phenotype determinations were based on the manufacturer guidelines. The strains were inoculated onto TSA plates and allowed to absorb onto agar for 10 min, and antibiotic discs were added after 24 h of incubation at 28 °C (Zhou et al., 2020).

**Expression Analysis of T3SS-related genes**

T3SS secretion was induced by Dulbecco's Modified Eagle Medium (DMEM) media (Nydam et al., 2014; Li et al., 2016), and strains HY9901 and HY9901Δ*vscB* were cultured for 12 h at 28 °C. The primers for T3SS related genes are shown in (Table 2). The genes in this study were *hop* (Guillon et al., 2010), *vopN*, *vscN* (Yuan et al., 2016), *vscO* (Zhou et al., 2013), and *vopS*, *vscL*, *vscK* (Nguyen et al., 2000). According to the experimental method of Li et al. (Li et al., 2016), total RNA was extracted using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instructions respectively. The first-strand cDNA was synthesized from the DNase I (Takara, Japan) treated total RNA using the Reverse Transcriptase M-MLV (Takara, Japan) according to the manufacturer's protocol. The PCR assay was performed in a 25 μL reaction volume containing 0.5 μL of each primer (10 μM), 2μL of diluted cDNA (1:10), 12.5 μL of 2 x TransStart$^{TM}$ Green qPCR SuperMix (TransGen, Beijing) and 9.5 μL sterile water. The PCR amplification procedure was carried out at 95 ℃ for 4 min, followed by 35 cycles of 95 ℃ for 20s, 50 ℃ for 20s and 72 ℃ for 20s. Melt curve analyses of amplification products were performed at the end of each PCR reaction. Samples were run in triplicate on the Bio-Rad iQ5 Real-time PCR System (Bio-Rad, CA, USA). 16S rRNA was used as an internal control. Each of the samples included six independent

individuals, respectively, to eliminate individual differences. All primer pairs amplified a single PCR product which was analyzed in triplicate using the appropriate Tm value by agarose gel electrophoresis and melting curve analysis.

**Transcriptome sequencing**

For transcriptome sequencing, HY9901 and Δ*vscB* cells were cultured in DMEM media (initial $OD_{600}$ of 0.01) at 28 °C for 12 h., The bacterial cells were harvested and then dissolved in TRIzol (Takara Bio, Inc.). The series of experiments, including mRNA extraction, RNA fragmentation, cDNA synthesis and RNA-Seq library construction, were conducted by Novogene Co., Ltd.

**Investigation of the viabilityof HY9901Δ*vscB* in vivo post vaccination**

The pearl gentian grouper injected intraperitoneally with 100μL $1\times10^5$cfu $mL^{-1}$HY9901Δ*vscB* extended to 7 days post vaccination. The organs including spleen and head-kidney were aseptically collected from day 1 to day 7. All the samples were weighed and homogenized in 1 ml PBS. The homogenates were serially diluted and plated in triplicate onto TCBS plates and incubated at 28℃ for 18 h. The bacteria counts were calculated by dividing the weights of the tissues and from the mean of three samples.

**Pearl gentian grouper vaccination and bacterial challenge**

Groupers were randomly divided into 2 groups with 85 fish in each group, and the water temperature was regulated at 26 °C using heating rods. The experimental vaccine group injected intraperitoneally with 100 μL Δ*vscB* bacterium solution (1 x $10^5$

CFU/mL) per tail. The control group was injected intraperitoneally with 100μL PBS per tail.

Four weeks post immunization, groupers (n=30) were anesthetized and challenged separately by IP inoculation of 100μL $1\times10^{8}$cfu $mL^{-1}$ of *V. alginolyticus* HY9901. The number of dead fish after HY9901 challenge was recorded for 16 days until the mortality rate stabilized. The dead fish were sampled and the bacteria in the diseased fish tissues were isolated. The bacteria were cultured on a TSA plate and identified by 16s rDNA sequencing to determine whether the death of grouper was caused by the challenge of *V. alginolyticus* HY9901.

The relative percent survival（RPS）was calculated according to the following formula: RPS (%) = (1-immunized group mortality / control group mortality) × 100%.

**Immune Gene Expression of grouper Induced by HY9901Δ*vscB* Vaccine**

Liver and spleen samples were taken from three fish from each group (state groups), 1 day prior to challenge. Immune-related gene expression levels were detected with real-time qPCR. Primers for IL-1β, MHC-1, IgM, TNF-α and CD8α are shown in Table 2, and β-actin was used as an internal reference gene. The procedures of RNA extraction, cDNA synthesis, and real-time qPCR for analysis of immune gene expression were carried out as previously described (Li et al., 2016).

**Statistical Analysis**

Statistical analyses were performed using SPSS 17.0 (SPSS Inc., USA)**.** Using the Student's t-test Group differences (strains HY9901 and HY9901Δ*vscB*) were determined by Duncan's test. ** indicates highly significant difference compared with

the control group ($p < 0.01$). * indicates significant difference compared with the control group ($p < 0.05$).

## Results

### Cloning, mutagenesis and complementation of *vscB* in *V. alginolyticus* HY9901

The *vscB* gene consisted of an open reading frame of 429 bp encoding 142 amino acids with a predicted molecular mass of 16.402 kDa and a theoretical isoelectric point of 5.48. The nucleotide sequence of HY9901 *vscB* was deposited in the GenBank database under the accession number MG905226 (**Fig.1**). Blast analysis (NCBI) of the deduced amino acid of *vscB* indicated that it has 60-91% identity with other *Vibrio spp.* and it shared the highest homology to *vscB* of *V. parahemolyticus* (91%) (**Fig. 2**).

To understand possible roles of VscB in *V. alginolyticus*, an unmarked *vscB* deletion mutant was constructed by using overlap PCR and a double-selection strategy. HY9901Δ*vscB* was confirmed by PCR by generating a fragment of 1396 bp (**Fig. 3A**). C-*vscB* was confirmed by PCR by generating a fragment of 587 bp (**Fig. 3B**).

### Characterization of the Δ*vscB*

Morphology was observed by transmission electron microscopy (TEM), and there was no discernible morphological difference among HY9901, Δ*vscB* and C-*vscB* (**Fig. 4**).

After 30 generations of continuous blind transmission of mutant Δ*vscB* a fragment of 974 bp was obtained by HY9901Δ*vscB* (**Fig. 5**).

There were no significant differences between growth rates of wild-type strain

309 HY9901, HY9901Δ*vscB* and C-*vscB* ($p > 0.05$) (**Fig. 6**). Also, no significant
310 differences were observed in biofilm formation in the HY9901, Δ*vscB* and C-*vscB*
311 strains ($p > 0.05$) (**Fig. 7**). The extracellular protease (ECPase) activity was not
312 significantly different in the HY9901, Δ*vscB* and C-*vscB* ($p > 0.05$) (**Table 3**). With
313 regards to swarming activity, the swarming circle of the wild strain HY9901was 32.22
314 ±0.07 mm, Δ*vscB* was 27.83 ±0.06 mm, and C-*vscB* was 31.18 ±0.06 mm (**Table 3**).
315 The swarming circle diameter of Δ*vscB* was smaller than that of wild strain HY9901
316 and C-*vscB*, indicating that the swarming ability was related to *vscB*. Grouper from
317 quarantined stocks recognized as disease free (Xu et al., 2011) were used (Sullivan and
318 Kim, 2008) to assess the virulence of the strain HY9901, Δ*vscB* and C-*vscB*. The
319 HY9901 Δ *vscB* mutant had an attenuated swarming phenotype and a 23-fold decrease
320 in virulence to grouper. ($p<0.01$, **Table 3**). The results showed that the virulence of *V.*
321 *alginolyticus* Δ*vscB* was significantly reduced.
322
323 **Antibiotic susceptibility**
324 All the strains were susceptible to amikacin, minocyline, teracyline, gentamicin
325 and doxycyciline; and resistant to cefperazone, oxacillin, clindamycin, ceftazidime,
326 penicillin, ampicillin, caebenicillin, cfazolin, ceftriaxone, cephradine, piperacillin,
327 cefuroxime, SMZ/TMP, aoxacin, and fuazzolidone. Both wild and mutant strains were
328 susceptible to chloramphenicol, neomycin and resistant to erythromycin, but C-*vscB*
329 was susceptible to erythromycin and resistant to chloramphenicol and neomycin. The
330 wild and C-*vscB* were susceptible to kanamycin, the mutant Δ*vscB* was resistant to

kanamycin (**Table 4**).

**T3SS-related genes expression analysis**

As the *vscB* gene regulates T3SS-related transcription levels, qRT-PCR was employed to analyze the transcription levels of T3SS-related genes including *vscL*, *vscK, vopN, vscO, vscN, vopS* and *hop. vscL* and *vscK* are apparatus proteins, *vopS*, *vopN, vscN* and *hop* are effector or regulatory proteins, *vscO* is a chaperone escort protein. The results showed that compared with HY9901 wild type, HY9901Δ*vscB* decreased the expression of *vopN, vscO*, while increasing the expression of *vopS, vscK, vscL, hop,* and *vscN*. (**Fig. 8**).

**Transcriptome sequencing analysis of HY9901 and strain Δ*vscB***

A total of 95 differentially expressed genes were screened by transcriptome sequencing analysis of HY9901 and strain Δ*vscB*, among which 57 genes were up-regulated and 38 genes were down-regulated, respectively (Table S1). GO analysis showed that the differentially expressed genes of HY9901 and strain Δ*vscB* were mainly associated with oxidation-reduction process, single-organism metabolic process and oxidoreductase activity (**Fig. 9).**

**Investigation of the viability of HY9901Δ*vscB* in vivo**

HY9901Δ*vscB* was able to disseminate into but survive transiently in fish head-kidney and spleen then was gradually eliminated from the host body **(Fig. 10)**. The highest bacterial number was detected in spleen on day 3, followed by the head-kidney.

## Vaccine efficacy

The protective efficacy of Δ*vscB* was evaluated by challenging grouper with *V. alginolyticus* wild strain 28 d after vaccinating fish with PBS or the Δ*vscB* strain. The results showed that the survival rate (RPS) in the Δ*vscB* group was 77.6%（**Fig.11**. The results indicated that Δ*vscB* had only minimal virulence and induced a protective response in grouper thus potentiating it's use as an attenuated live vaccine.

## Immune gene expression of grouper induced by HY9901Δ*vscB* vaccine

The immune response of grouper immunized with the HY9901Δ*vscB* live attenuated vaccine was assessed by qPCR to analyze the transcriptional levels of pro-inflammatory and immunoglobulin-related immune genes. The results showed that the group vaccinated with the mutant strain HY9901Δ*vscB* had significantly increased expression of MHC-Iα and CD8α genes in the liver and spleen compared to control fish injected with PBS ($p<0.01$). (**Fig. 12**)

## Discussion

The type III secretion system of *V. alginolyticus* is a determining factor in the process of infecting the host cell. Molecular chaperones play an important role in this system. The secretion of effector proteins required the assistance of chaperone proteins to retain the secretory capacity of the effector protein (Day et al., 2003). *yscB* is the chaperone of the effector *NosA* in *Yersinia*, whereby it can change the morphology of host cell after invasion (Day et al., 2003), but little is known about the *vscB* of *V.*

*alginolyticus*.

In the current study, we knocked out the T3SS gene *vscB* of *V. alginolyticus*, explored the physiology and pathogenicity of the mutant, and evaluated its efficacy as a live attenuated vaccine. In the genetic stability test, the Δ*vscB* strain was still capable of amplifying the upstream and downstream fusion fragments of *vscB* after 30 generations, indicating that the genetic information of the Δ*vscB* can be stably transmitted to offspring.

Production of extracellular products (ECP) mainly including protease, hemolysin and siderophore, are thought to be characteristics of virulent strains of *V. alginolyticus* (Fletcher, 1977; Lee et al., 1997). Biofilm formation is a multicellular behavior by which bacteria colonize the surface of host tissues, leading to resistance to antibiotics and host immune responses (Parsek and Singh, 2003; Verstraeten et al., 2008). Nevertheless, our results indicated that there was no significant difference between HY9901 and Δ*vscB* in morphology, growth, biofilm, and ECPase activity. Therefore, *vscB* may not be responsible for these characteristics in *V. alginolyticus.* The flagella contributed to swarming motility and facilitated bacterial access to appropriate niches inside the host after *Vibrio* infection (Watnick et al., 2001). Studies have shown that the flagella is essential for virulence, flagellum formation, efficient motility and symbiotic competence during initial squid light organ colonization by *Vibrio* (Millikan et al., 2004). In the present study, the Δ*vscB* mutant of *V. alginolyticus* had suppressed swarming motility. The results suggested that *vscB* is a positive contributor to swarming motility in *V. alginolyticus* and might function indirectly through regulating the

399 expression level of flagella, however this needs further investigation. The $LD_{50}$ of
400 Δ*vscB* was 23.2 times lower than that of wild strain. These results indicated that the
401 *vscB* gene contributes to the pathogenesis of *V. alginolyticus.*

402 According to the experimental results, the mutant strain Δ*vscB* was more resistant
403 to drugs than the wild strain HY9901, so it could be speculated that the *vscB* gene might
404 be related to drug resistance genes.

405 Zhou et al. found that *sycD, vopB* and *vopD* mRNAs decreased significantly in the
406 Δ*vscO* mutant compared with the wild-type strain (Zhou et al., 2013). Chen found that
407 the expression levels of *vscX* mRNA in the deletion strain Δ*vscO* were significantly up-
408 regulated at a late growth stage (Chen et al., 2017). In this study, Δ*vscB* had decreased
409 expression of *vopN* and *vscO* and increased expression of *hop*, *vscN, vscK, vscL* and
410 *vopS.* These results suggested that T3SS-related genes could play an important role in
411 mediating these proteins and regulating the transcription of these T3SS genes to
412 maintain a suitable level of protein synthesis and secretion via an unclear mechanism.
413 However, the regulatory mechanism network of *V. alginolyticus* T3SS is still unknown
414 and its role in pathogenesis warrants further attention.

415 At present, there is no commercial Vibriosis vaccine for grouper in China,
416 although the disease has been causing great economic losses to the aquaculture industry
417 (Li et al., 2010; Xie et al., 2005). Virulence gene deletion is becoming a common
418 strategy for attenuated live vaccine development, which benefits from its low toxicity
419 and sustainable protection. In recent years, live attenuated vaccines have demonstrated
420 great potential in preventing and controlling lethal bacterial diseases (Pang et al., 2018;

Li et al., 2015; Wang et al., 2014). Pang et al. found that grouper vaccinated via IP injection with HY9901Δ*hop* induced a high antibody titer with a RPS value of 84% after challenging with the wild type *V. alginolyticus* HY9901 (Pang et al., 2018). Chen et al. showed that the Δ*acfA* mutant caused a high antibody titer with a RPS value of 81.1% after challenging with *V. alginolyticus* HY9901 in pearl gentian grouper (Chen et al., 2019). Chen et al. also found that Δ*sodB* induced a high antibody titer and provided valid protection with a RPS value of 86.5% without inducing clinical symptoms after challenging with *V. alginolyticus* HY9901 (Chen et al., 2019). Zhang et al. showed that the Δ*yscB* mutant elicited a higher antibody titer and provided protective efficacy against both subcutaneous and intranasal *Y. pestis* challenge (Zhang et al., 2013). In this study, the RPS of grouper vaccinated with Δ*vscB* mutant reached 77.6%, which was significantly higher than that of the control group. The results showed that the Δ*vscB* mutant could provide effective protection against challenge with *V. alginolyticus* and has the potential as an attenuated live vaccine.

It has already been confirmed that live attenuated vaccines can induce a more robust humoral and cell-mediated immune response than killed bacteria (Killeen et al., 2001). The increase of MHC-Iα expression in the spleens was also found in pearl gentian grouper vaccinated with a *V. alginolyticus acfA* mutant (Chen et al., 2019). IgM gene expression can be induced by intraperitoneal injection with *Yersinia ruckeri* in rainbow trout (Deshmukh et al., 2013). CD8 is the specific marker of cellular defense, and is increased in fish vaccinated with an *Edwardsiella tarda* mutant in turbot (Xiao et al., 2013). In this study, the elevated expression of immune-related genes (MHC-Iα,

IgM and CD8α), confirmed the stimulation of innate and acquired immune responses in pearl gentian grouper. Future work using immunohistochemical methods or flow cytometry sorting rather than qPCR will further provide a deeper understanding of the protective immune mechanisms of HY9901Δ*vscB* in pearl gentian grouper or other fish.

In conclusion, we have successfully constructed an in-frame deletion strain of HY9901Δ*vscB* and investigated its biological characteristics and pathogenicity revealing suppressed swarming motility and virulence despite stable morphology, growth, biofilm and ECPase activity consistent with the wild-type strain. The *vscB* gene regulates T3SS proteins and drug resistance genes, and a Δ*vscB* mutant strain provides protection to immunized grouper. There was no significant difference between HY9901 and HY9901Δ*vscB* in morphology, growth, biofilm, and ECPase activity. The Δ*vscB* mutant of *V. alginolyticus* had suppressed swarming motility. The $LD_{50}$ of HY9901Δ*vscB* was 23.2 times lower than that of wild strain. The *vscB* gene regulated T3SS proteins and drug resistance genes. The Δ*vscB* mutant induced a high level of protection against *V. alginolyticus* challenge. Taken together, the results enhance our understanding of the role of *vscB* in the biology and pathogenicity of *V. alginolyticus* and offer an insight into the development of a live attenuated vaccine for preventing Vibriosis.

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## Competing interests

The authors declare that there is no conflict of interest.

## References

Bauer A.W., Kirby W.M., Sherris J.C., Turck M., 1966. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol. 45(4), 493-496.

Bennett J.C., Hughes C., 2000. From flagellum assembly to virulence: the extended family of type III export chaperones. Trends in microbiology. 8(5), 202-204. https://doi.org /10.1016/ S0966-842X (00)01751-0.

Birtalan S.C., Phillips R.M., Ghosh P., 2002. Three-dimensional secretion signals in chaperone-effector complexes of bacterial pathogens. Molecular cell. 9(5), 971-980. https://doi.org/ 10.1016/s1097-2765(02)00529-4.

Cai S.H., Wu Z.H., Jian J.C., Lu Y.S., 2007. Cloning and expression of the gene encoding an extracellular alkaline serine protease from Vibrio alginolyticus strain HY9901, the causative agent of vibriosis in Lutjanus erythopterus (Bloch). Journal of fish diseases. 30(8), 493-500. https://doi.org/10.1111/j.1365-2761.2007.00835.x.

Cai S.H., Wu Z.H., Jian J.C., Lu Y.S., 2007. Cloning and expression of gene encoding the thermostable direct hemolysin from Vibrio alginolyticus strain HY9901, the causative agent of vibriosis of crimson snapper (Lutjanus erythopterus). Journal of applied microbiology. 103(2), 289-296. https://doi.org/10.1111/j.1365-2672.2006.03250.x.

Cai X.H., Wang B., Peng Y.H., Li Y., Lu Y.S., Huang Y.C., Jian J.C., Wu Z.H, 2017. Construction of a Streptococcus agalactiae phoB mutant and evaluation of its potential as an attenuated modified live vaccine in golden pompano, *Trachinotus ovatus*. Fish & Shellfish immunology. 63, 405-416. https://doi.org/10.1016/j.fsi.2016.11.050.

Chen X.Y., Hu C.Q., Ren C.H., Chen C., 2002. Development, characterization and application of monoclonal antibody against Vibrio alginolyticus lipopolysaccharide. High Technol. 12(11), 90-95.

Chen X.Y., Li j., Pang H.Y., Chang Y.S., Huang Y.C., Wu Z.H., Jian J.C., 2017. Molecular Cloning, Bioinformatics Analysis and Expression Analysis of Type III Secretion System( T3SS) Injectisome

Gene vscX from Vibrio alginolyticus. Agricultural Biotechnology. 6(01), 41-45. https://doi.org/10.19759/j.cnki.2164-4993.2017.01.010.

Chen Y.Y., Cai S.H., Jian J.C., 2019. Protection against Vibrio alginolyticus in pearl gentian grouper ( ♀ Epinephelus fuscoguttatus × ♂ Epinephelus lanceolatu ) immunized with an acfA-deletion live attenuated vaccine. Fish & Shellfish Immunology. 86, 875-881. https://doi.org/ 10.1016/j.fsi.2018.12.030.

Chen Y.Y., Wu F.L., Pang H.Y., Tang J.F., Cai S.H., Jian J.C., 2019. Superoxide dismutase B (sodB), an important virulence factor of Vibrio alginolyticus, contributes to antioxidative stress and its potential application for live attenuated vaccine. Fish & shellfish immunology. 89, 354-360. https://doi.org/10.1016/j.fsi.2019.03.061.

Cornelis G.R., Van Gijsegem F., 2000. Assembly and function of type III secretory systems. Annual Reviews in Microbiology. 54(1), 735-774. https://doi.org/10.1146/annurev.micro.54.1.735.

Day J.B., Ferracci F., Plano G.V., 2003. Translocation of YopE and YopN into eukaryotic cells by Yersinia pestis yopN, tyeA, sycN, yscB and lcrG deletion mutants measured using a phosphorylatable peptide tag and phosphospecific antibodies. Molecular Microbiology. 47(3), 807–823. https://doi.org/10.1046/j.1365-2958.2003.03343.x.

Day J.B., Plano G.V., 1998. A complex composed of SycN and YscB functions as a specific chaperone for YopN in Yersinia pestis. Molecular microbiology. 30(4), 777-788. https://doi.org/ 10.1046/j.1365-2958.1998.01110.x.

Deshmukh S., Kania P.W., Chettri J.K., Skov J., Bojesen A.M., Dalsgaard I., Buchmann K., 2013. Insight from molecular, pathological, and immunohistochemical studies on cellular and humoral mechanisms responsible for vaccine-induced protection of rainbow trout against *Yersinia ruckeri*. Clinical and vaccine immunology. 20(10), 1623-1641. https://doi.org/10.1128/CVI.00404-13.

Fletcher M., 1977. The effects of culture concentration and age, time, and temperature on bacterial attachment to polystyrene. Can J Microbiol. 23(1), 1-6. https://doi.org/10.1139/m77-001.

Galán J.E., Collmer A., 1999. Type III secretion machines: bacterial devices for protein delivery into host cells. Science. 284(5418), 1322-1328. https://doi.org/10.1126/science.284.5418.1322.

Guillon H., Eb F., Mammeri H., 2010. Characterization of CSP-1, a novel extended-spectrum β-lactamase produced by a clinical isolate of Capnocytophaga sputigena. Antimicrobial agents and chemotherapy. 54(5), 2231-2234. https://doi.org/10.1128/AAC.00791-09.

Haldar S., 2012. Vibrio Related Diseases in Aquaculture and Development of Rapid and Accurate Identification Methods. Journal of Marine Science Research & Development. 3(1), 1-7. https://doi.org/10.4172/2155-9910.S1-002.

Kierek K., Watnick P.I., 2003. Environmental determinants of Vibrio cholerae biofilm development. Appl Environ Microbiol. 69(9), 5079-5088. https://doi.org/10.1128/aem.69.9.5079-5088.2003.

Killeen K.P., V.J., 2001. DiRita, Live attenuated bacterial vaccines. New Vaccine Technol. 151.

Lafferty K.D., Harvell C.D., Conrad J.M., Friedman C.S., Kent M.L., Kuris A.M., Powell E.N., Rondeau D., Saksida S.M., 2015. Infectious diseases affect marine fisheries and aquaculture economics. Annual Review of Marine Science. 7(1), 471-496. https://doi.org/ 10.1146/annurev-marine-010814-015646.

Lee K.K., Yu S.R., Liu P.C., 1997. Alkaline serine protease is an exotoxin of Vibrio alginolyticus in kuruma prawn, Penaeus japonicus. Curr Microbiol. 34(2), 110-117. https://doi.org/ 10.1007/s002849900153.

Li J., Mo Z.L., Li G.Y., Xiao P., Huang J., 2015. Generation and evaluation of virulence attenuated

543 mutants of Edwardsiella tarda as vaccine candidates to combat edwardsiellosis in flounder
544 (Paralichthys olivaceus). Fish & shellfish immunology. 43(1), 175-180. https://doi.org/
545 10.1016/j.fsi.2014.12.018.
546 Li N.Q., Yang Z.H., Bai J.J., Fu X.Z., Liu L.H., Shi C.B., Wu S.Q., 2010. A shared antigen among Vibrio
547 species: outer membrane protein-OmpK as a versatile Vibriosis vaccine candidate in Orange-spotted
548 grouper (Epinephelus coioides). Fish & shellfish immunology. 28(5-6), 952-956.
549 https://doi.org/10.1016/j.fsi.2010.02.010.
550 Li P., Rivera-Cancel G., Kinch L.N., Salomon D., Tomchick D.R., Grishin N.V., Orth K., 2016. Bile salt
551 receptor complex activates a pathogenic type III secretion system. Elife 5, e15718.
552 https://doi.org/10.7554/eLife.15718.
553 Li W.X., Yao Z.J., Sun L.N., Hu W.J., Cao J.J., Lin W.X., Lin X.M., 2016. Proteomics analysis reveals a
554 potential antibiotic cocktail therapy strategy for Aeromonas hydrophila infection in biofilm. Journal
555 of proteome research. 15(6), 1810-1820. https://doi.org/10.1021/acs.jproteome.5b01127.
556 López J.R., De La Roca E., Núñez S., De La Herran R., Navas J.I., Manchado M., Herrera M., Toranzo
557 A.E., 2009. Identification of Vibrio harveyi isolated from diseased cultured wedge sole
558 Dicologoglossa cuneata. Diseases of Aquatic Organisms. 84(3), 209-217. https://doi.org
559 /10.3354/dao02045.
560 Mahbub K.R., Paul K.P., Ahmed M.M., 2011. Prevalence of Vibrio spp and antibiogram of isolates from
561 shrimp rearing ponds in Bangladesh. Journal of Advanced Scientific Research. 2(4), 74-80.
562 Millikan D.S., Ruby E.G., 2004. Vibrio fischeri flagellin A is essential for normal motility and for
563 symbiotic competence during initial squid light organ colonization. J. Bacteriol. 186(13), 4315-4325.
564 https://doi.org/10.1128/JB.186.13.4315-4325.2004.
565 Nguyen L., Paulsen I.T., Tchieu J., Hueck C.J., Saier Jr M.H., 2000. Phylogenetic analyses of the
566 constituents of type III protein secretion systems. J. Mol. Microbiol. Biotechnol. 2(2), 125-144.
567 Nydam S.D., Shah D.H., Call D.R., 2014. Transcriptome analysis of Vibrio parahaemolyticus in type III
568 secretion system 1 inducing conditions. Front Cell Infect Microbiol. 4, 1. https://
569 doi.org/10.3389/fcimb.2014.00001.
570 Pang H.Y., Qiu M.S., Zhao J.M., Hoare R., Monaghan S.J., Song D.W., Chang Y.S., Jian J.C., 2018.
571 Construction of a Vibrio alginolyticus hopPmaJ (hop) mutant and evaluation of its potential as a
572 live attenuated vaccine in orange-spotted grouper (Epinephelus coioides). Fish & shellfish
573 immunology. 76, 93-100. https://doi.org/10.1016/j.fsi.2018.02.012.
574 Parsek M.R., Singh P.K., 2003. Bacterial biofilms: an emerging link to disease pathogenesis. Annu Rev
575 Microbiol. 57(1), 677-701. https://doi.org/10.1146/annurev.micro.57.030502.090720.
576 Reed L.J. Muench H., 1938. A simple method of estimating fifty per cent endpoints. American Journal
577 of Epidemiology. 27(3), 493-497. https://doi.org/10.1093/oxfordjournals.aje.a118408.
578 Sadok K., Mejdi S., Nourhen S., Amina B., 2013. Phenotypic characterization and RAPD fingerprinting
579 of Vibrio parahaemolyticus and Vibrio alginolyticus isolated during Tunisian fish farm outbreaks.
580 Folia Microbiol. 58(1), 17–26. https://doi.org/10.1007/s12223-012-0174-x.
581 Spaeth K.E., Chen Y.S., Valdivia R.H., 2009. The Chlamydia type III secretion system C-ring engages a
582 chaperone-effector protein complex. PLoS pathogens. 5(9). https://doi.org/
583 10.1371/journal.ppat.1000579.
584 Stebbins C.E., Galán J.E., 2001. Maintenance of an unfolded polypeptide by a cognate chaperone in
585 bacterial type III secretion. Nature. 414(6859), 77-81. https://doi.org/10.1038/35102073.
586 Sullivan C., Kim C.H., 2008. Zebrafish as a model for infectious disease and immune function. Fish &

shellfish immunology. 25(4), 341-350. https://doi.org/10.1016/j.fsi.2008.05.005.
Verstraeten N., Braeken K., Debkumari B., Fauvart M., Fransaer J., Vermant J., Michiels J., 2008. Living on a surface: swarming and biofilm formation. Trends Microbiol. 16(10), 496-506. https://doi.org/10.1016/j.tim.2008.07.004.
Wang J., Zou L.L., Li A.X., 2014. Construction of a Streptococcus iniae sortase A mutant and evaluation of its potential as an attenuated modified live vaccine in Nile tilapia (Oreochromis niloticus). Fish & shellfish immunology. 40(2), 392-398. https://doi.org/10.1016/j.fsi.2014.07.028.
Wang Q.Y., Liu Q., Cao X.D., Yang M.J., Zhang Y.X., 2008. Characterization of two TonB systems in marine fish pathogen Vibrio alginolyticus: their roles in iron utilization and virulence. Archives of microbiology. 190(5), 595-603. https://doi.org/10.1007/s00203-008-0407-1.
Watnick P.I., Lauriano C.M., Klose K.E., Croal L., Kolter R., 2001. The absence of a flagellum leads to altered colony morphology, biofilm development and virulence in Vibrio cholerae O139. Mol Microbiol. 39(2), 223-35. https://doi.org/10.1046/j.1365-2958.2001.02195.x.
Wattiau P., Bernier B., Deslée P., Michiels T., Cornelis G.R., 1994. Individual chaperones required for Yop secretion by Yersinia. Proceedings of the National Academy of Sciences. 91(22), 10493-10497. https://doi.org/10.1073/pnas.91.22.10493.
Wattiau P., Woestyn S., Cornelis G.R., 1996. Customized secretion chaperones in pathogenic bacteria. Molecular microbiology. 20(2), 255-262. https://doi.org/10.1111/j.1365-2958.1996.tb02614.x.
Wei G.B., Cai S.H., Wu Y.Z., Ma S.H., Huang Y.C., 2020. Immune effect of Vibrio harveyi formalin-killed cells vaccine combined with chitosan oligosaccharide and astragalus polysaccharides in ♀ Epinephelus fuscoguttatus × ♂ Epinephelus lanceolatus. Fish & shellfish immunology. 98, 186-192. https://doi.org/10.1016/j.fsi.2020.01.015.
Windle H.J., Kelleher D., 1997. Identification and characterization of a metalloprotease activity from Helicobacter pylori. Infect Immun. 65(8), 3132-3137. https://doi.org/ 10.1128/IAI.65.8.3132-3137.1997.
Xiao J.F., Chen T., Liu B., Yang W.Z., Wang Q.Y., Qu J.B., Zhang Y.X., 2013. Edwardsiella tarda mutant disrupted in type III secretion system and chorismic acid synthesis and cured of a plasmid as a live attenuated vaccine in turbot. Fish & Shellfish immunology. 35(3), 632-641. https://doi.org/10.1016/j.fsi.2013.05.022.
Xie Z.Y., Hu C.Q., Chen C., Zhang L.P., Ren C.H., 2005. Investigation of seven Vibrio virulence genes among Vibrio alginolyticus and Vibrio parahaemolyticus strains from the coastal mariculture systems in Guangdong, China. Lett Appl Microbiol. 41(2), 202-207. https://doi.org/ 10.1111/j.1472-765X.2005.01688.x.
Xu Z.N., Wang Y., Han Y., Chen J.X., Zhang X.H., 2011. Mutation of a novel virulence-related gene mltD in Vibrio anguillarum enhances lethality in zebra fish. Research in microbiology. 162(2), 144-150. https://doi.org/10.1016/j.resmic.2010.08.003.
Yao G., Tan Y.X., Zou W.Z., Xu X.J., Xing Y.L., Ji R.X., Zheng T.L., Yan Q.P., 2012. Characteristics of Vibrio alginolyticus biofilm formation. Fisheries Science. 31(2), 73-78. https://doi.org/ 10.16378/j.cnki.1003-1111.2012.02.003.
Young G.M., Smith M.J., Minnich S.A., Miller V.L., 1999. The Yersinia enterocolitica motility master regulatory operon, flhDC, is required for flagellin production, swimming motility, and swarming motility. Journal of bacteriology. 181(9), 2823-2833. https://doi.org/ 10.1128/JB.181.9.2823-2833.1999.
Yuan Y.H., Pang H.Y., Zhou Z.J., Song D.W., Ding Y., Jian J.C., Wu Z.H, 2016. Construction of Live

Attenuated Vaccine against Vibrio alginolyticus and the Evaluation on Its Immunoprotection Effect. Animal Husbandry and Feed Science. 8(02), 85-89+93. https://doi.org/ 10.19578/j.cnki.ahfs.2016.02.007.

Zhang X.C., Qi Z.Z., Du Z.M., Bi Y.J., Zhang Q.W., Tan Y.F., Yang H.Y., Xin Y.Q., Yang R.F., Wang X.Y., 2013. A live attenuated strain of Yersinia pestis ΔyscB provides protection against bubonic and pneumonic plagues in mouse model. Vaccine. 31(22), 2539-2542. https://doi.org/10.1016/j.vaccine.2013.03.054.

Zhao Z., Chen C., Hu C.Q., Ren C.H., Zhao J.J., Zhang L.P., Jiang X., Luo P., Wang Q.B., 2010. The type III secretion system of Vibrio alginolyticus induces rapid apoptosis, cell rounding and osmotic lysis of fish cells. Microbiology. 156(9), 2864-2872. https://doi.org/ 10.1099/mic.0.040626-0.

Zhou S.H., Tu X.T., Pang H.Y., Hoare R., Monaghan S.J., Luo J.J, Jian J.C., 2020. A T3SS Regulator Mutant of Vibrio alginolyticus Affects Antibiotic Susceptibilities and Provides Significant Protection to Danio rerio as a Live Attenuated Vaccine. Frontiers in Cellular and Infection Microbiology. 10, 183. https://doi.org/10.3389/fcimb.2020.00183.

Zhou Z.J., Pang H.Y., Ding Y., Cai J., Huang Y.C., Jian J.C., Wu Z.H., 2013. VscO, a putative T3SS chaperone escort of Vibrio alginolyticus, contributes to virulence in fish and is a target for vaccine development. Fish & shellfish immunology. 35(5), 1523-1531. https://doi.org/ 10.1016/j.fsi.2013.08.017.

**Table 1 Bacterial strains and plasmids used in this study**

| Strains, Plasmids | Relevant information | Source or references |
|---|---|---|
| *V. alginolyticus* HY9901 | Wild type, isolated from diseased *Lutjanus sanguineus* off the Southern China coast | Cai et al., 2007 |

| | | |
|---|---|---|
| Δ*vscB* | HY9901 carrying an in-frame deletion of *vcBS* | This study |
| C-vscB | HY9901 Δ*vscB* containing plasmid of pBAD33cm-*vscB* | This study |
| *E. coli* DH5α | Competent cells | Sangon 2018 |
| *E.coli* β2163 | Competent cells | This study |
| pMD18-T | Cloning vector, Ampr | TakaRa 2018 |
| pBAD33-CM | *E.coli-suicide vector* | This study |
| pLP12 | *E.coli*-suicide vector | This study |

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678 **Table 2 Sequences of primers used in this study.**

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| Primer name | Primer sequence(5'-3') | Accession number or references |
|---|---|---|
| Cloning primers | | |

| | | |
|---|---|---|
| vscB-F1 | ATGTTAGATAAGATGATGAAATC | MG905226 |
| vscB-R1 | TCATGGTAACCACACTGTATG | |
| muntant construction | | |
| *vscB* -MF1 | GGAATCTAGACCTTGAGTCGGCTATCAGGGGAAGACACCAG | GU074526.1 |
| *vscB*-MR1 | TGTATGGTTGATTGTGTGGTTCACCATCTTATCTAACATTTAACGCCC | |
| *vscB*-MF2 | GGGCGTTAAATGTTAGATAAGATGGTGAACCACACAATCAACCATAC | |
| *vscB* MR2 | ACAGCTAGCGACGATATGTC GGAGGACCCGCAAGATAGAC | |
| *vscB*-TF | ACTACATCAACTCTCGTCTGGGC | |
| *vscB*-TR | AACTTCTTTGCTAGACGCACTCG | |
| pLP-UF | GACACAGTTGTAACTGGTCCA | |
| pLP-UR | CAGGAACACTTAACGGCTGAC | |
| Complement construction | | |
| pBAD30-ZF | CTAGAGTCGACCTGCAGGCA | GU074526.1 |
| pBAD30-ZR | AGCTCGAATTCGCTAGCCCA | |
| vscB-RF | TGGGCTAGCGAATTCGAGCTAGGAGGAATTCACCATGTTAGATAAGATGATG | |
| vscB-RR | CATGCCTGCAGGTCGACTCTAGTCATGGTAACCACACTGTATG | |
| RP4-F2 | CGAATTGGGTACCAGCGCTT | |
| RP4-R2 | TACCGTCGACGCCGGCCAGC | |
| pBAD30-mcf-TF | CCATAAGATTAGCGGATCCTACCT | |
| pBAD30-mcf-TR | CTTCTCTCATCCGCCAAAACAG | |
| 16S-F | TTGCGAGAGTGAGCGAATCC | (Zhou et al.,2020) |
| 16S -R | ATGGTGTGACGGGCGGTGTG | |
| VopN-F | TGAACTCGTTTCGGACTA | |
| VopN-R | ACTTTCTGGACTCGCACT | |
| hop-F | CTTCGCTTTCGGTTTGCT | |
| hop-R | AATACCATCCCACCCTGT | |
| VscO-F | GAGCTGGAAACATTAAGACA | |
| VscO-R | TTGCTGCAACTGAACGAA | |
| VscN-F | TAGGCGAAGAAGGAATGG | |
| VscN-R | GCGATAGAAGTGGCAACAA | |
| VscK-F | GGCGTTATCTCCCGTTCC | |
| VscK-R | CTCCGCCCACCATCAATA | |
| VscL-F | TACCACGGTGAGTGTAGTTC | |
| VscL-R | CGTAACCGACTTCAGGGA | |
| VopS-F | AGTTTTGGAAGTGTTAGCG | |
| VopS-R | ACATTGCCTCTGTCATCG | |
| β-actin-F | GGACAGCTACGTTGGTGATGA | (Chenyanyan,2019) |
| β-actin-R | GGACAGCTACGTTGGTGATGA | |
| IL-1β-F | TCTGGGCATCAAGGGCACACA | |
| IL-1β-R | CCATGTCGCTGTTCGGATCGA | |
| TNF-α-F | GCCACAGGATCTGGCGCTACTC | |

| | |
|---|---|
| TNF-α-R | CTTCCGTCGCTGTCCTCATGTG |
| IgM-F | TACAGCCTCTGGATTAGACATTAG |
| IgM-R | CTGCTGTCTGCTGTTGTCTGTGGAG |
| CD8α-F | GCTGGTGATTCTGCTGATTTG |
| CD8α-R | GGACTTGGAGGATGACTTTAGG |
| MHC-Iα-F | GCCGCCACGCTACAGGTTTCTA |
| MHC-Iα-R | TCCATCGTGGTTGGGGATGATC |

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698 **Table 3 Characteristics of different strains**

| Characteristics | HY9901 | Δ*vscB* | C-*vscB* |
|---|---|---|---|
| Activity of ECPase [a] | 0.06 ± 0.02 | 0.06 ± 0.01 | 0.06 ± 0.02 |
| Swarming(mm)[b] | 32.22 ± 0.07 | 27.83 ± 0.06 | 31.18 ± 0.06 |
| $LD_{50}$(cfu/mL)[c] | $7.29 \times 10^4$ | $1.69 \times 10^{6}$** | $9.05 \times 10^4$ |

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700 **: $p<0.01$.

701 Values are mean ± standard deviation for three trials.

702 [a] Bacteria were incubated in TSB for 18 h at 28℃.

703 [b] Swarming diameters were measured after 24 h incubation at 28℃ on TSA
704 containing 0.3% agar plates.

705 [c] $LD_{50}$ were evaluated in pearl gentian grouper with an average weight of 20.0 ± 2g.

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**Table 4 Drug sensitivity test results of the HY9901, Δ*vscB* and *C-vscB***

| Antibiotic | Dose (μg) | Bacteriostatic circle diameter (mm) | | | | | |
|---|---|---|---|---|---|---|---|
| | | HY9901 | Sensitivity | Δ*vscB* | Sensitivity | *C-vscB* | Sensitivity[a] |
| Cefperazone | 75 | 0 | R | 0 | R | 0 | R |
| Oxacillin | 1 | 0 | R | 0 | R | 0 | R |
| Clindamycin | 2 | 0 | R | 0 | R | 0 | R |
| Ceftazidime | 30 | 0 | R | 0 | R | 0 | R |
| Penicillin | 10U | 0 | R | 0 | R | 0 | R |
| Ampicillin | 100 | 0 | R | 0 | R | 0 | R |
| Caebenicilin | 100 | 0 | R | 0 | R | 0 | R |
| Cefazolin | 30 | 8.0±0.2 | R | 0 | R | 0 | R |
| Ceftriaxone | 30 | 9.3±0.3 | R | 0 | R | 0 | R |
| Cephradine | 30 | 0 | R | 0 | R | 0 | R |
| Piperacillin | 100 | 0 | R | 0 | R | 0 | R |
| Cefuroxime | 30 | 0 | R | 0 | R | 0 | R |
| SMZ/TMP | 23.75/1.25 | 0 | R | 0 | R | 0 | R |
| Aboren | 30 | 0 | R | 0 | R | 0 | R |
| Vancomycin | 30 | 0 | R | 0 | R | 10.0±0 | R |
| Cephalexin | 30 | 0 | R | 0 | R | 0 | R |
| Polymyxin B | 200IU | 0 | R | 8.2±0.2 | R | 0 | R |
| Norfloxadcin | 10 | 0 | R | 0 | R | 0 | R |
| Ofloxacin | 5 | 0 | R | 0 | R | 0 | R |
| Ciprofloxacin | 5 | 0 | R | 0 | R | 0 | R |
| Amikacin | 30 | 13.3±0.3 | I | 13.1±0.1 | I | 13.2±0.1 | I |
| Minocyline | 30 | 18.5±0.2 | S | 15.2±0.2 | I | 16.8±0.3 | I |
| Tetracyline | 30 | 13.5±0.2 | I | 13.0±0.2 | I | 14.8±0.2 | I |
| Gentamicin | 10 | 14.5±0.1 | I | 12.9±0.2 | I | 15.0±0.1 | I |
| Furazolidone | 300 | 10.5±0.4 | R | 10.0±0.1 | R | 10.0±0.1 | R |
| Chloramphenicol | 30 | 17.2±0.3 | S | 17.5±0.2 | S | 0 | R |
| Kanamycin | 30 | 14.1±0.2 | I | 12.3±0.2 | R | 14.5±0.2 | I |
| Erythromycin | 15 | 10.1±0.2 | R | 8.5±0.1 | R | 13.2±0.2 | I |
| Doxycycline | 30 | 16.5±0.3 | S | 14.2±0.1 | I | 16.6±0.2 | S |
| Neomycin | 30 | 14.2±0.3 | I | 12.6±0.3 | I | 0 | R |

[a]S(susceptible) I(intermediate) R(resistance)

## Figure Legends

**Figure 1: PCR products following cloning by PCR of *V. alginolyticus vscB* gene**

M: DL2000 DNA marker; 1-2: PCR products of *vscB*

**Figure 2: Multiple sequence alignments of *V. alginolyticus* HY9901 T3SS protein VscB.**

*V. alginolyticus* Accession NO.GU074526.1
*V. parahaemolyticus* Accession NO.KOY47612.1,
*V. campbellii* Accession NO.WP_045453226.1
*V. harveyi* Accession NO.WP_042605857.1
*V. tasmaniensis* Accession NO.WP_017096464.1
*V. jasicida* Accession NO.WP_045424028.1

**Figure 3: Construction and confirmation of the knockout mutant strain HY9901Δ*vscB* and C-*vscB***

(A)M: DL2000 marker; Lane 1. The 1396 bp fragment amplified from genomic DNAs of HY9901Δ*vscB* using primer pairs of vscB-TF / vscB-TR. Lane 2. *E. coli β2163*（pLP12-*vscB*）using primer pairs of vscB-TF / vscB-TR. Lane 3.The 1771bp fragment amplified from genomic DNAs of the wild-type strain HY9901using primer pairs of vscB-TF / vscB-TR
(B) M: DL2000 marker; Lane 1-7. The 587 bp fragment amplified from genomic DNAs of C-*vscB* using primer pairs of pBAD-mcf-TF/pNAD-mcf-TR

**Figure 4: Transmission electron microscopy (TEM) of the morphological features of *Vibrio alginolyticus* HY9901 (A) and HY9901Δ*vscB* (B).**

A:HY9901 B:C-*vscB* C:Δ*vscB*

**Figure 5: Hereditary stability of *Vibrio alginolyticus* Δ*vscB***

M: DL 2 000 DNA marker;
1--4: The 974 bp fragment amplified from genomic DNAs of HY9901Δ*vscB* using primer pairs of vscB-MF1 / vscB-MR2

**Figure 6: Growth features of *Vibrio alginolyticus* HY9901, Δ*vscB* and *C-vscB***

Aliquots of cell culture were taken at various time points and measured for cell density at $OD_{600}$.

**Figure 7: Measurement of *Vibrio alginolyticus* biofilm by crystal violet ammonium oxalate .**

**Figure 8: Expression of induced *Vibrio alginolyticus* HY9901 and HY9901Δ*vscB* T3SS-related genes**
**By DMEM.**

HY9901Δ*vscB* had decreased the expression of *vopN*($p$<0.01), *vscO*($p$<0.05), while increase the expression of *vopS*($p$<0.01), *vscK*($p$<0.01), *vscL*($p$<0.01), *hop*($p$<0.05), and *vscN*($p$<0.05).

**Figure 9: The most enriched GO terms of differentially expressed genes**

**Figure 10 Propagation of HY9901Δ*vscB* in grouper kidney (A) and spleen (B) following i.p. injection with 100μL $1\times10^5$cfu $mL^{-1}$Δ*vscB*. Control fish were i.p. injection with 100 μL sterile PBS. The number of viable bacteria was shown as the mean ± standard of three samples.**

**Figure 11：Survival in groups vaccinated with HY9901Δ*vscB* and PBS following challenge with *Vibio alginolyticus* HY9901.**

Control group were injected intraperitoneally with PBS.

**Figure 12: Comparative analysis of the expression of immune-related genes in liver and spleen of grouper given the live attenuated** HY9901ΔvscB ***Vibrio alginolyticus*** **vaccine vs. unvaccinated grouper.**

A: liver of grouper B: spleen of grouper

The liver and spleen of grouper were sampled at 1 day before challenge, and the mRNA level of each immune-related gene was normalized to that of β-actin expression. Bars represent the mean relative expression of three biological replicates and error bars represent standard deviation. The group vaccinated with the mutant strain HY9901ΔvscB had significantly increased expression of MHC-I, TNF-α, IgM, and CD8αgenes both in liver and spleen compared to control fish injected with PBS($p<0.01$).

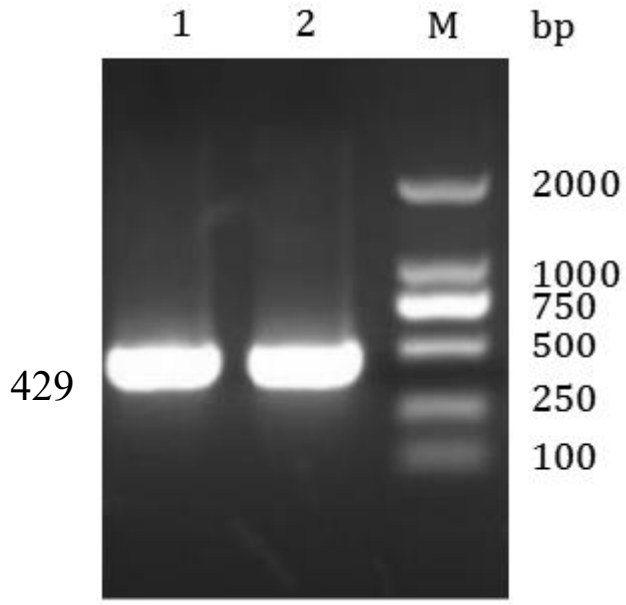
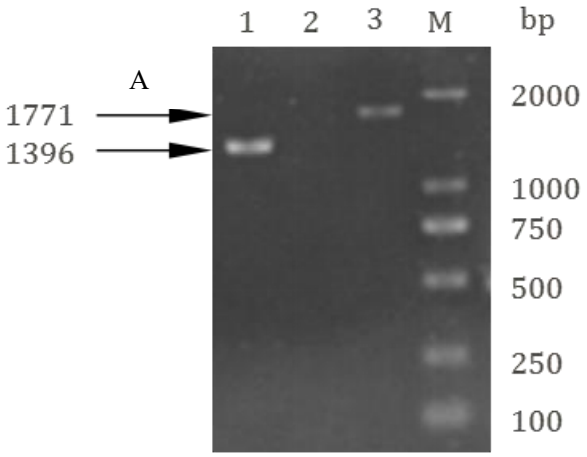



Figure 1


V.alginolyticus : MLDKMMKSLAEALKVGDFIASENGSYNIEVDQLSLTIKQHASWFLWEATLPFQFKEHLDYQKEQALQRCMQLS : 73
V.parahaemolyticus : MLDKMMKSLAETLKIGDFIASENGSYNIEVDQLSLTIKQHASWFLWEAALPFQFKEHLDYQKEQALQRCMQLS : 73
V.campbellii : MLDQMMKSLAATLGVGDFIASNNGSYDVEVDQMLLNIKQHSSWFLWETVLPFQFEQHLDYQKEQALKHCMQLS : 73
V.harveyi : MLDQMMKSLAATLGVGDFIASNNGSYDIEVDQMLLNIKQHSSWFLWETVLPFQFEQHLDYQKEQTLKHCMLLS : 73
V.tasmaniensis : MLDKMMKSLAETLREGDFIASSNGSYDIEVDQMQLNISQHSSWFLWETVLPFYFESHLDFQKEQALKHCMQLS : 73
V.jasicida : MLDQMMKSLAATLGVGDFIASNNGSYDIEIDQMLLNIKQHSSWFLWETVLPFQFEQHLDYQKEQTLKQCMQLS : 73
identity
V.alginolyticus : LKTIREDGGVLTTNDLQQLILQSKVRVEDCSVERFSALLSKHVNLCERYIALLEQARVNHTINHTVWLP : 142 100%
V.parahaemolyticus : LKTIREDGCVLTANDLQQLILQSKVRIEDCSIELFSSLLSKHVNLCERYMALLEQERVNHTINHNVWLP : 142 91%
V.campbellii : LKTLRDTSSTLTVNDVEQLIVQGKMNMESATAEALCAQLSQHVNLVEQFSGVLEHQRVNHTVSHSIWIP : 142 61%
V.harveyi : LKTLRDTSSTLTVNDVEQLIVQGKMNMESVTTEALCAQLSQHVNLVEQFSGVLEHQRVNHTVNHSIWIP : 142 61%
V.tasmaniensis : LKTLRDTRSTLTVNDKEQLIMQGKMTMESATTEELCTQLAQHVNLVEQFNDLLEHQRVNHTVSHSVWIP : 142 61%
V.jasicida : LKTLRDTSSTLTVNDVEQLIVQGKMNMESVTTEALCAQLSQHVNLVEQFSGVLEHQRVNHTVSHSIWIP : 142 60%

Figure 2


1 2 3 M bp
A
1771
1396
2000
1000
750
500
250
100

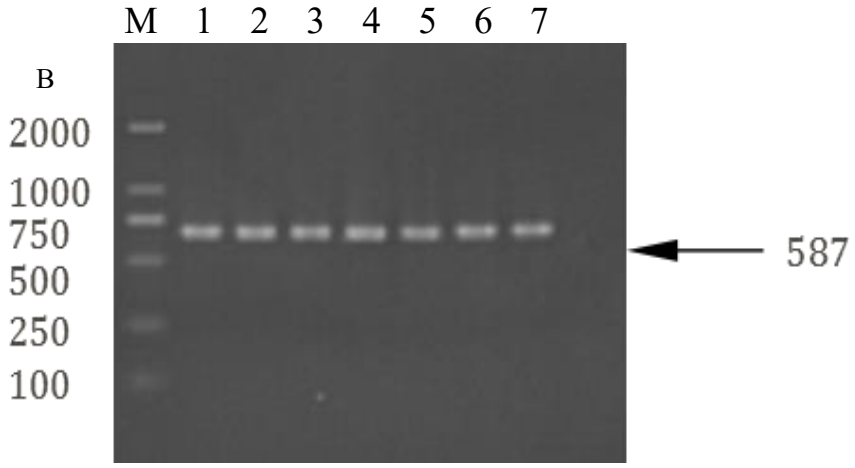

M 1 2 3 4 5 6 7
B
2000
1000
750
500
250
100
587

Figure 3

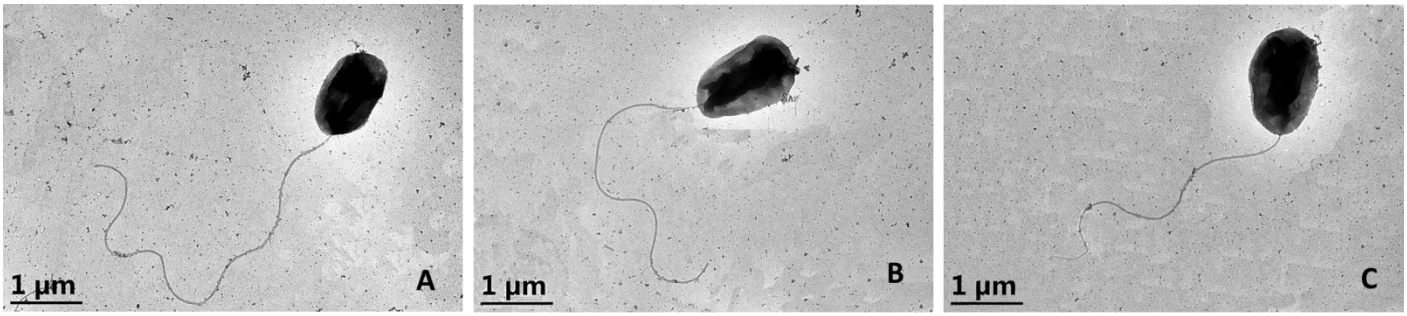



Figure 4

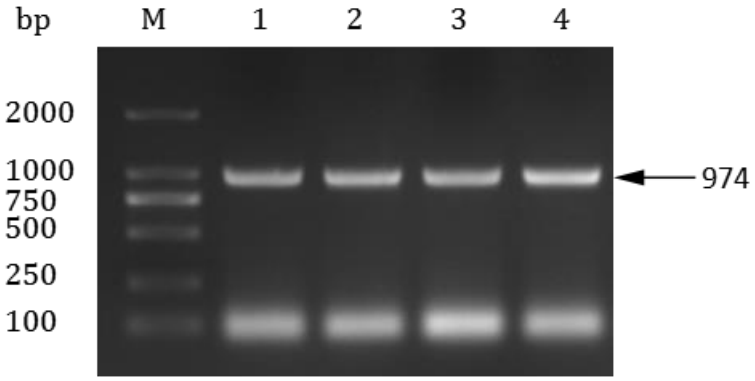



Figure 5

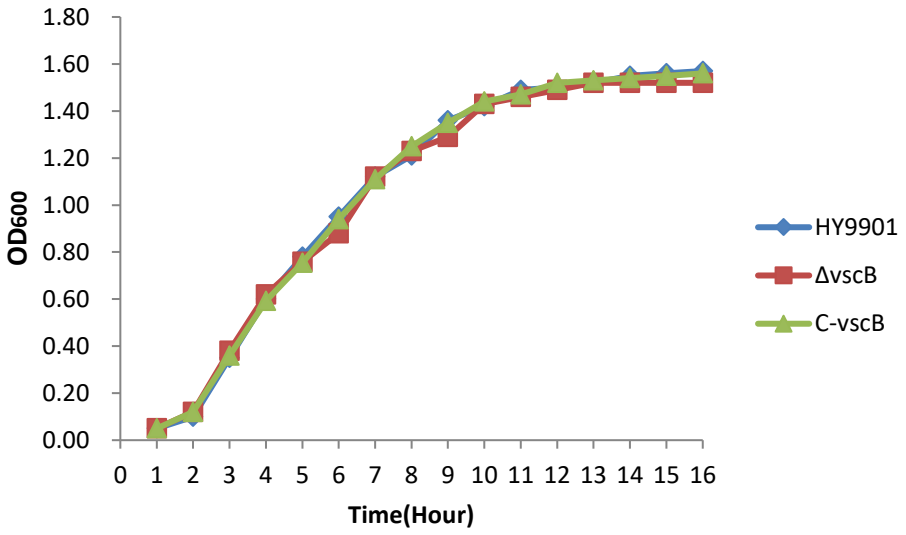

1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
OD600
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Time(Hour)
HY9901
ΔvscB
C-vscB

Figure 6

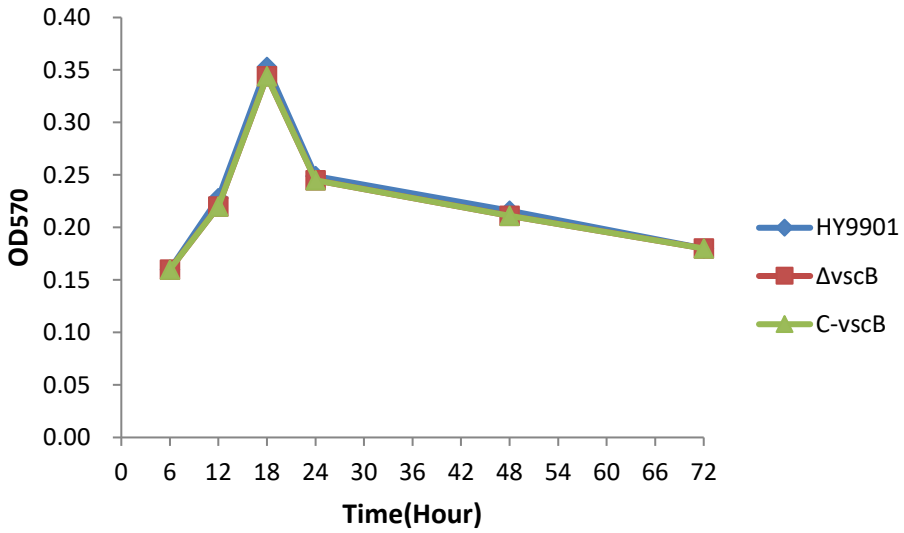

0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
OD570
0
6
12
18
24
30
36
42
48
54
60
66
72
Time(Hour)
HY9901
ΔvscB
C-vscB

Figure 7

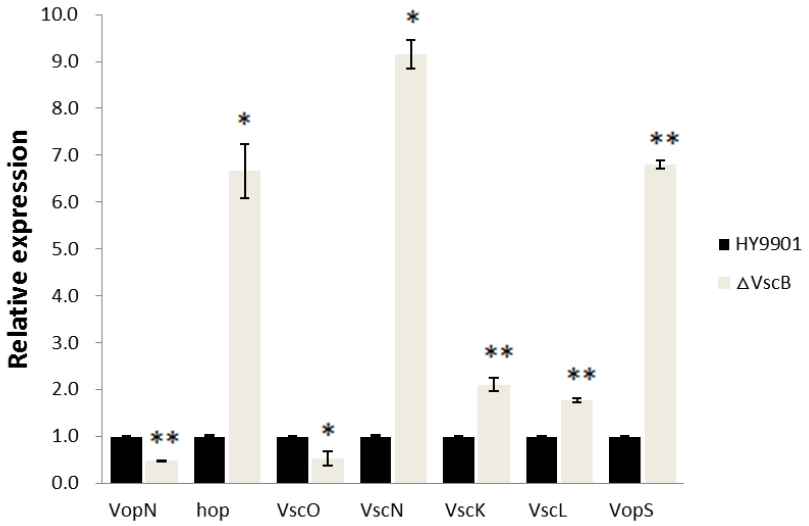

Relative expression
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
VopN
hop
VscO
VscN
VscK
VscL
VopS
HY9901
ΔVscB

Figure 8

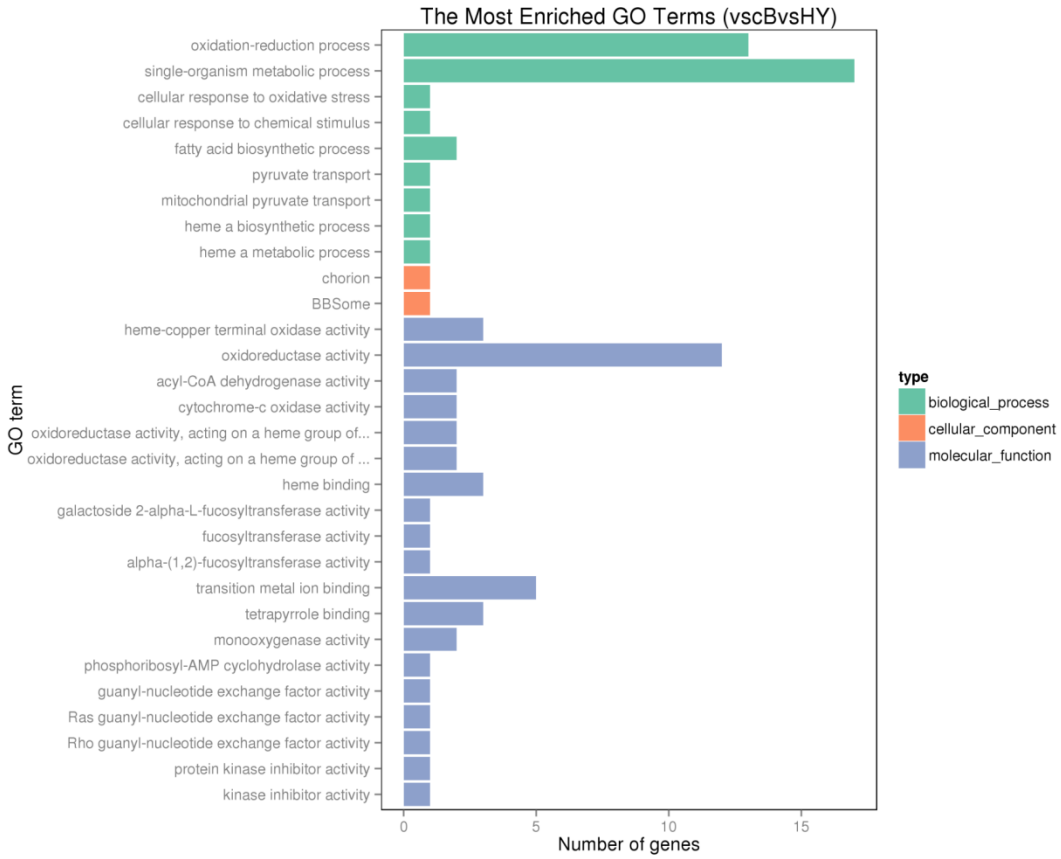
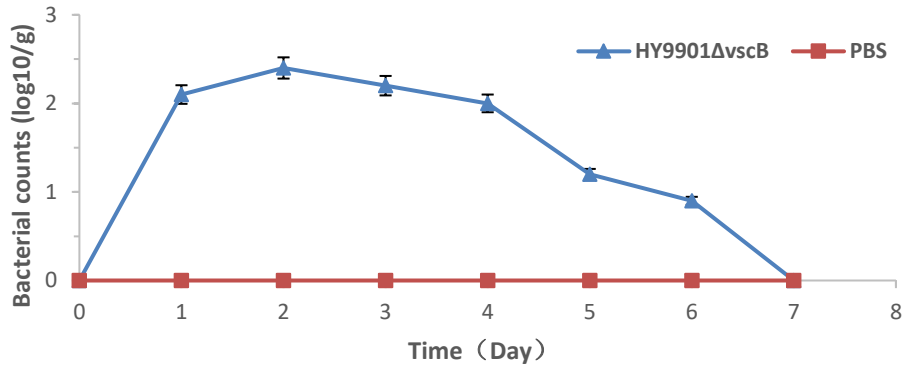

The Most Enriched GO Terms (vscBvsHY)
oxidation-reduction process
single-organism metabolic process
cellular response to oxidative stress
cellular response to chemical stimulus
fatty acid biosynthetic process
pyruvate transport
mitochondrial pyruvate transport
heme a biosynthetic process
heme a metabolic process
chorion
BBSome
heme-copper terminal oxidase activity
oxidoreductase activity
acyl-CoA dehydrogenase activity
cytochrome-c oxidase activity
oxidoreductase activity, acting on a heme group of...
oxidoreductase activity, acting on a heme group of ...
heme binding
galactoside 2-alpha-L-fucosyltransferase activity
fucosyltransferase activity
alpha-(1,2)-fucosyltransferase activity
transition metal ion binding
tetrapyrrole binding
monooxygenase activity
phosphoribosyl-AMP cyclohydrolase activity
guanyl-nucleotide exchange factor activity
Ras guanyl-nucleotide exchange factor activity
Rho guanyl-nucleotide exchange factor activity
protein kinase inhibitor activity
kinase inhibitor activity
GO term
0
5
10
15
Number of genes
type
biological_process
cellular_component
molecular_function

Figure 9

A


HY9901ΔvscB
PBS
Bacterial counts (log10/g)
Time（Day）
0
1
2
3
4
5
6
7
8

B

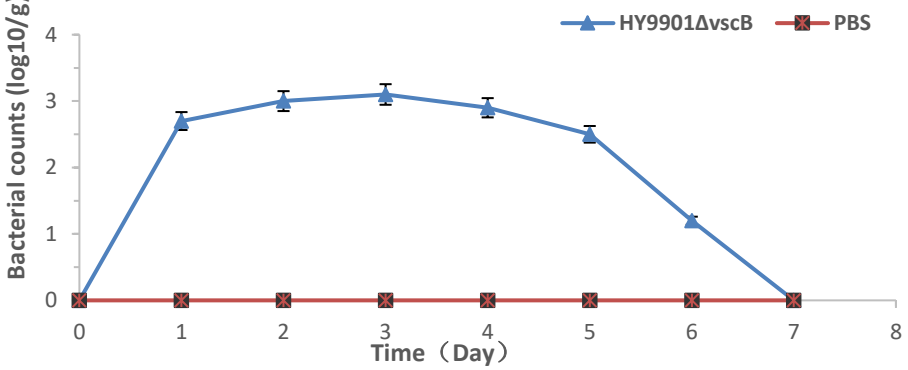

HY9901ΔvscB
PBS
Bacterial counts (log10/g)
Time（Day）
0
1
2
3
4
5
6
7
8

Figure 10

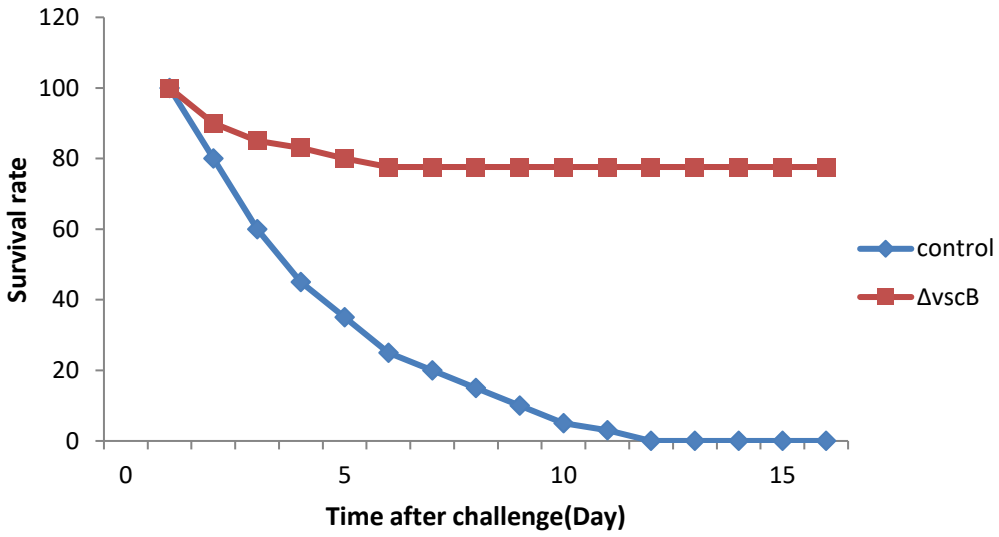
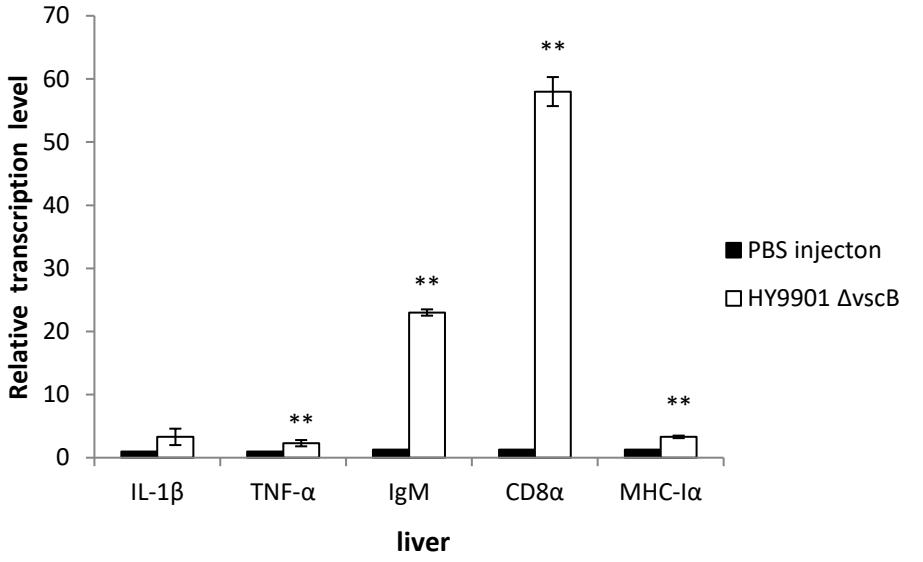
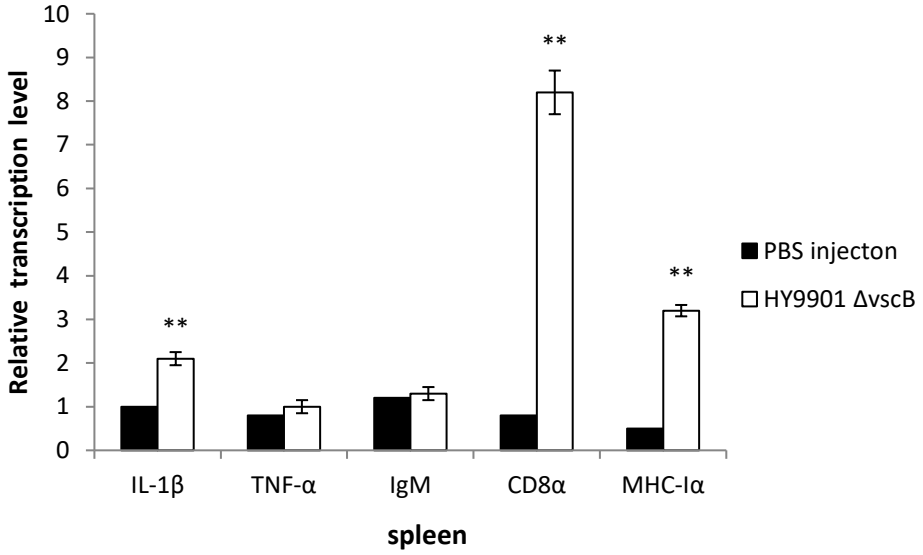

120
100
80
60
40
20
0
Survival rate
0
5
10
15
Time after challenge(Day)
control
ΔvscB

Figure 11




1090




1091

1092 Figure 12