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Superoxide dismutase B (*sodB*), an important virulence factor of *Vibrio alginolyticus*, contributes to antioxidative stress and its potential application for live attenuated vaccine

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ABSTRACT

Vibrio alginolyticus is an opportunistic and halophilic Gram-negative pathogen in limiting the development of aquatic industry and affecting human health. SODs are oxidative enzymes that play a critical role in oxidative defense. In this study, an in-frame deleted mutant strain (Δ*sodB*) was constructed by allelic exchange mutagenesis to investigate physiological role of *sodB* in pathogenicity of *V. alginolyticus*. The results exhibited that Δ*sodB* showed no differences in growth compared with wild-type strain HY9901 (WT), but led to increasing in biofilm formation, ECPase activity and sensitivity to hydrogen peroxide, decreasing in swarming motility, adherence to CIK cells, SOD activity and virulence. In addition, Δ*sodB* induced a high antibody titer and provided a valid protection with a relative percent survival value of 86.5% without inducing clinical symptoms after challenging with WT. These results suggest that *sodB* is important for normal physiological function, oxidation resistance and virulence in *V. alginolyticus*, and Δ*sodB* may be considered as an effective live attenuated vaccine against *V. alginolyticus*.

1. Introduction

In recent years, vibriosis caused by *V. alginolyticus* frequently and seriously influenced the healthy development of the aquaculture industry with the deterioration of the aquatic environment [1,2]. Symptoms of fish infected with vibriosis includes tissue necrosis, skin ulceration, gastroenteritis and inflammatory reactions [3–5]. Sometimes *V. alginolyticus* infects human. There were sporadic cases that human were infected by seafood and water contaminated with *V. alginolyticus* [6–8].

Reactive oxygen species (ROS) can be produced not only in the metabolic process of bacteria, but also in the host's immune response to pathogens [9,10]. Inflammation induced by *V. alginolyticus* generally results in mass generation of ROS. Although stimulated by a large amount of ROS, *V. alginolyticus* was able to successfully colonize and survive in the intestine, indicating that it has an effective system to adapt to oxidative stress [11,12]. The regulation of ROS is influenced by antioxidant enzymes and small molecule antioxidants, and superoxide dismutase (SOD) plays an important role in the removal of ROS [13]. Three types of SODs are identified based on their prosthetic metal cofactors, including MnSOD, FeSOD, and CuZnSOD, encoded by the *sodA*, *sodB*, and *sodC* genes, respectively. In *Pseudomonas aeruginosa*, FeSOD is more important than MnSOD for aerobic growth, resistance to paraquat, and optimal pyocyanin biosynthesis [14]. The *sodB* has been shown to be essential for bacterial persistence and virulence during host infection, and *sodB*-deficient mutants of *Francisella tularensis* demonstrated hypersensitivity to oxidative stress and attenuated virulence in mice [15]. However, there is little study about the role of *sodB* in *V. alginolyticus*.

Gene knockout is becoming a routine technique for site-directed mutagenesis to construct a vaccine candidate with the development of vaccines in a recent decade [16–18]. It is a virulence-attenuated mutated pathogen which possesses the merit of weaken pathogenicity and a long-term or lifelong protection without clinical symptoms and obvious pathological changes in vaccinated animals. Several attenuated live vaccines have been proved to achieve a higher survival rate of fish, and to induce prominent humoral and cell-mediated immune response [16,18,19].

In the study, to understand the role of *sodB* in *V. alginolyticus*, a *sodB*-deletion mutant (Δ*sodB*) was constructed to observe and analyze

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Table 1
Bacterial strains, plasmids, cell line and primers used in this study.

Strain, Plasmid, Cell line and Primer	Relevant imformation or sequence (5′-3′)	Source
Bacterial Strains		
V. alginolyticus HY9901	Wild type strain	Cai et al., 2007a
Δ*sodB*	*V. alginolyticus* HY9901, in-frame deletion of $sodB_{58\text{-}561}$	This study
E. coli DH5α	Competent cells	TakaRa
E. coli β2163	Competent cells	Maibo Bioscience
Cell line		
CIK	Kidney cell of grass carp	Bioleaf
Plasmids		
pLP12	*E. coli*- suicide vector	This study
pBAD33cm-rp4	*E. coli*- suicide vector	This study
Primers	Nucleotide sequence (5′-3′)	
SodB-UF	GGAATCTAGACCTTGAGTCGTACTTTGGTAACTCAGCACGGATA	This study
SodB -UR	TTACTTCGCTAGGTTCTCAGCTACCGCGTAAGGAAGAGCTGGTAG	This study
SodB-DF	CTACCAGCTCTTCCTTACGCGGTAGCTGAGAACCTAGCGAAGTAA	This study
SodB-DR	ACAGCTAGCGACGATATGTCAGTCAGCGCATCTGGTTTTAGG	This study
SodB -TF	TTAGCTTGAATTTGATGCTGACG	This study
SodB -TR	CTGCGCCTTAATACCGATCTGG	This study
pBAD30-ZF	CTAGAGTCGACCTGCAGGCA	This study
pBAD30-ZR	AGCTCGAATTCGCTAGCCCA	This study
SodB-RF	TGGGCTAGCGAATTCGAGCTAGGAGGAATTCACCATGGCATTTGAA	This study
SodB-RR	TGCCTGCAGGTCGACTCTAGTTACTTCGCTAGGTTCTC	This study
PBAD-mcf-TF	CCATAAGATTAGCGGATCCTACCT	This study
PBAD-mcf-TR	CTTCTCTCATCCGCCAAAACAG	This study

the physiological characteristics, SOD activity and sensibility to hydrogen peroxide. Moreover, fish vaccination, antibody test and histological analysis were performed to evaluate the potential of strain Δ*sodB* as live attenuated vaccine.

2. Materials and methods

2.1. Bacterial strains and fish

Bacterial strains and the plasmids used in this study are listed in Table 1. *V. alginolyticus* strain HY9901 (WT) was isolated from diseased fish *Lutjanus erythopterus* in Zhanjiang harbor area of Guangdong province, China, and cultured with Tryptic Soy Broth (TSB, Haling, China) at 28 °C. *Escherichia coli* DH5α was cultured with Luria-Bertani broth (LB, Haling, China).

Healthy pearl gentian grouper (♀*Epinephelus fuscoguttatus* × ♂*Epinephelus lanceolatu*) weight (60 ± 2 g) were purchased from a fish farm in Dongnan Harbor (Guangdong Province, China). Fish are considered healthy by sera agglutination and bacteriological recovery tests described by Pang et al. [17]. The groupers were grown in 500 l containers with constant aeration and a temperature of 28 ± 1 °C. Fish were fed twice a day with a formulated pellet (No. 4c grouper feed, manufactured by Dongwan Feed Group, Zhanjiang, China). Pearl gentian grouper were acclimated to the indoor laboratory condition for 2 weeks before experiment. Groupers were anaesthetized with tricaine methane sulfonate (MS222) (Kuer, Anwei, China) before injection and sample collection. Animal experiments were complied with ethical standards and approved by Guangdong Provincial Key Laboratory of Pathogenic Biology and Epidemiology for Aquatic Economic Animals Ethics Committee.

2.2. Construction of ΔsodB and complementation of the mutant

In this study, Δ*sodB* was constructed with allelic exchange mutagenesis. Firstly, two specific pairs of primers were designed to obtain the *sodB* upstream homologous arm fragment A (Primers: SodB-UF and SodB-UR) and downstream homologous arm fragment B (Primers: SodB-DF and SodB-DR). And then, fragment A and B are used as templates, and the AB fragments are fused together by overlapping PCR. The AB fusion fragment was connected to the suicide vector pLP12, and the recombinant product was transformed into the *E. coli* DH5α. The positive clone was selected and the recombinant plasmid plP12-SodB was extracted and converted into *E. coli* β2163. The positive clones were selected and used for the conjugation with *V. alginolyticus*, and Δ*sodB* was successfully constructed with a PCR identification (Primers: SodB-TF and SodB-TR) after twice homologous recombination.

The *sodB* fragment (Primers: SodB -RF and SodB -RR) and pBAD33cm-rp4 vector fragment (Primers: pBAD30-ZF and pBAD30-ZR) were amplified, connected and transformed into *E. coli* DH5α. The recombinant plasmid was extracted from DH5α and transformed into *E. coli* β2163. Positive clones were selected (Primers: pBAD-mcf-TF and pBAD-mcf-TR) and sequenced to confirm the successful construction of *sodB* complementation strain (C-*sodB*).

2.3. Characterization of ΔsodB

The Δ*sodB* phenotype was characterized by cell morphology, growth ability, biofilm formation, extracellular protease (ECPase) activity, swarming motility, adherence rate and LD_{50} test. Briefly, WT and Δ*sodB* were cultured in TSB for 18 h, and cell morphology was observed by transmission electron microscope. Growth ability was measured using the method of Zhou et al. [18]. Biofilm formation was measured using the crystal violet stain method described by Kierek and Watnick [20] and observed by laser scanning confocal microscopy. Extracellular protease (ECPase) activity was carried out according to the method of Windle and Kelleher [21]. Swarming motility was measured using the method described by Young et al. [22]. The cell adherence was performed using the method of Cai et al. [16]. CIK cells grown in 24-well plates were infected with WT, Δ*sodB* and C-*sodB*. The plates were incubated for 2 h at 28 °C with 5% CO_2. Cells were then washed with PBS and digested by utilizing trypsin enzyme. Serial dilutions of the mixture were plated onto TSA agar to calculate adhering bacteria. The results were showed as the adherence rate relative to that of WT set as 100%. All of experiments were performed in triplicate.

LD_{50} values of WT and Δ*sodB* were evaluated to assess the virulence to the healthy grouper. Eighty fish was divided into 4 groups, and then 20 fish of each group were injected intraperitoneally with 0.1 ml WT, Δ*sodB* or C-*sodB* suspended in sterile phosphate buffered saline (PBS) containing 10^5-10^9 cfu/ml respectively after anaesthetized by immersion in a 20 mg/l solution of MS-222. The negative control was treated

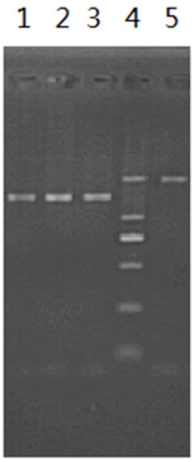

Fig. 1. PCR identification of *V. alginolyticus* strain Δ*sodB* (Primers: SodB-TF and SodB-TR). 1–3: PCR amplifications with Δ*sodB* genomic DNA; 4: 2, 000 DNA marker; 5: PCR amplifications with wild type HY9901 genomic DNA.

by the injection of 0.1 ml sterile PBS. The fish were monitored for 14 days, and any dead fish were removed for the routine bacteriological examination. The experiment was duplicated twice, and LD_{50} values were calculated via the approach of Reed and Muench [23].

2.4. Sensitivity to hydrogen peroxide (H_2O_2)

To detect sensitivity to H_2O_2, WT, Δ*sodB* and C-*sodB* was grown respectively in TSB to an OD_{600} of 0.2, then H_2O_2 were added at concentration of 0, 0.1 and 1 mM/l. All strains continued to be cultured at 28 °C for 24 h in a shaking incubator.

2.5. SOD enzyme activity assay

The assay was carried out to detect the changes of SOD activity of Δ*sodB* compared with WT and C-*sodB*. Strains were disrupted by ultrasonication following washing with PBS three times. The homogenates were centrifuged at 4000 r for 10 min at 4 °C, and the supernatants were used for enzyme activity assays. Total protein was measured following Bradford's method [24] with BSA as a standard. The activity of SOD was determined using kits purchased from Nanjing Jiangcheng Bioengineering Institute (Nanjing, China). The assay was performed in triplicate. All enzymatic activities are expressed in U/mg.

2.6. Vaccination and analysis of antibody levels

Vaccination experiments were carried out to evaluate the potential of *ΔsodB* as an attenuated live vaccine candidate against *V. alginolyticus*. 80 pearl gentian grouper were divided into PBS group (control group) and Δ*sodB* group (the treated group). They were immunized intraperitoneally with 100 μl sterilized PBS and 100 μl 6.9×10^5 CFU/ml strain Δ*sodB*, respectively. The experiment was duplicated twice.

Serum were collected to measure antibody levels using ELISA during the first to eighth week post-vaccination. The 96-hole ELISA plate was coated with 100 μl of formalin-killed *V. alginolyticus* by overnight incubation at 4 °C. Two-fold serial dilutions of the fish serum samples were added to the 96-hole plate blocked with 2% BSA. Antibody binding to the antigen was detected using mouse anti-pearl gentian grouper IgM polyclonal antibody. Plates were incubated with goat anti-mouse IgG-HRP conjugates. The reaction was developed with the 3,3,5,5-tetramethylbenzidine (TMB) substrate with H_2O_2 and stopped with 2 M H_2SO_4. Optical density was measured at 450 nm using a micro plate reader. The correct antibody value was diluted multiples corresponded with the highest ELISA OD values.

2.7. Bacterial challenge

After 56 days post-immunization, the pearl gentian grouper were challenged intraperitoneally with 100 μl 8.1×10^8 CFU/ml WT. The cumulative mortality was monitored for 14 days post-challenge. The experiment was duplicated twice. The relative percent survival (RPS) was calculated according to the following formula: RPS = [1 - (mortality of vaccinated fish/mortality of control fish)] × 100%. The bacteria were identified by re-isolating from the liver, kidney and spleen tissues of all dead fishes at the end of the experiment.

2.8. Histopathological sections post-vaccination

After 56 d post-immunization, in order to evaluate the safety of attenuated Δ *sodB*, the spleens and livers were collected from the PBS group and the Δ *sodB* group and used to perform histological examination. Samples of WT group of LD_{50} experiment (2.3) were used as pathogen positive control. Spleens and livers tissues were fixed in Davidson's Fixative (Shanghai Tarui Bioscience) for 24 h. Then the tissues were orderly dehydrated in ethanol with different concentrations (50%→70%→80%→90%→95%→100%), equilibrated with xylene, and embedded in paraffin. Paraffin block were then cut to 5 μm thickness, stained by Haematoxylin–Eosine and examined for pathological alterations with an optical microscope.

2.9. Statistical analysis

The results were examined with a one - way analysis of variance (ANOVA) using the SPSS 19.0 computer program (SPSS Inc., Chicago, USA). Mean comparisons were tested using Duncan's test. Data are presented as mean ± SE and subjected to Student's t - test for determining significant differences between groups. The differences were considered significant if $p < 0.05$.

3. Results

3.1. Identification and characteristics of the ΔsodB mutant

An isogenic *sodB* mutant of the wild strain HY9901 was constructed through allelic exchange mutagenesis. The result was confirmed by a PCR identification (Fig. 1) and direct DNA sequencing (data not shown) and showed a *sodB*-deletion mutant (Δ*sodB*) was successfully constructed without alteration of the remaining sequence.

3.2. Physiological characteristics

Several physiological phenotypes were compared among WT, Δ*sodB*, and C-*sodB*. Morphology was observed by transmission electron microscope, and there was no discernible morphological difference among WT, Δ*sodB* and C-*sodB* (Fig. 2). The growth curves of WT and Δ*sodB* was drawn and showed no significant difference between WT and Δ*sodB* (Fig. 3). The Δ*sodB* (about 98 μm) had significantly enhanced biofilm forming when compared with WT (about 32 μm) and C-*sodB* (about 28 μm) at 24 h ($p < 0.05$, Fig. 4A–C). However, no difference can be seen among WT, Δ*sodB* and C-*sodB* at 48 h and 72 h during the

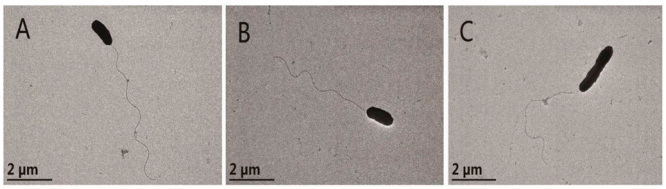

Fig. 2. Morphological characteristcs of WT strain (A), Δ*sodB* strain (B) and C-*sodB* strain(C) via transmission electron microscope.

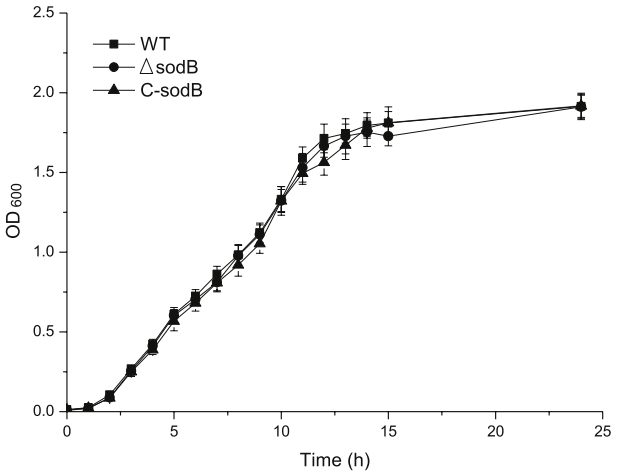

Fig. 3. The growth curve of WT, Δ*sodB* and C-*sodB*.

incubation (Fig. 4D). A smaller swarming diameter (4.3 ± 0.5 mm) and higher activity of ECPase (0.64 ± 0.04) was detected in Δ*sodB* than that of WT, and adherence rate of Δ*sodB* was about 3-folds lower than that of the WT (Table 2).

In LD_{50} experiment, The LD_{50} value in WT was 7.56×10^6 cfu/fish, while it was9.32×10^7 cfu/fish in Δ*sodB*, indicating there is significantly different in the virulence between the Δ*sodB* and WT ($p < 0.01$, Table 2), and the virulence of the complementary strain C-*sodB* was similar to that of WT. The dead fish exhibited clinical symptoms of vibriosis characterized by hemorrhaging, swelling, and ulcers on the skin surface. None of fish died or became diseased in the control group.

3.3. Sensitivity to H_2O_2 and SOD enzyme activity

The strains were grown in the presence of H_2O_2 to evaluated its antioxidant capacity by growth characteristics determination. The results showed that WT and C-Δ*sodB* grown normally at the 0.1 mM concentration of H_2O_2, and grown slowly when the concentration of H_2O_2 was 1 mM (Fig. 5). However, the growth of Δ*sodB* was suppressed and significantly lower than that of WT and C-Δ*sodB* in all concentration of H_2O_2. The results of SOD activity showed that SOD activities of Δ*sodB* decreased significantly during 4–24 h compared with WT and C-*sodB* (Fig. 6).

Table 2
Characteristics of *V. alginolyticus* WT strain, Δ*sodB* strain and C-*sodB* strain.

Characteristics	WT	Δ*sodB*	C-*sodB*
swarming motiliy (mm)	25.7 ± 0.3	4.3 ± 0.5*	19.8 ± 0.2
activity of ECPase	0.38 ± 0.01	0.64 ± 0.04*	0.36 ± 0.02
adherence rate (%)	0.46 ± 0.04	0.16 ± 0.03*	0.34 ± 0.05
LD_{50}	7.56×10^6	9.32×10^7*	9.24×10^6

Values are expressed as the mean ± SE. The asterisks indicated significant differences (*, $p < 0.05$) among the groups.

3.4. Measurement of antibody levels

The antibody level of grouper immunized with PBS (control) and Δ*sodB* was assessed by ELISA during the first to eighth week after vaccination. The results showed that antibodies could be detected at the first week after vaccination in Δ*sodB* group. The antibody titer of Δ*sodB* group firstly increased during the first to fourth week and then declined during the fifth to eighth week. During the first to eighth week, $\log_2$ (antibody titers) in the sera of Δ*sodB* group all reached above 4.0, and the maximum reached 11.0, while that of the control group was only 1.0 to 3.0 at all times (Fig. 7). The specific antibody titers immunized with Δ*sodB* was significantly higher than that in the PBS group ($p < 0.05$).

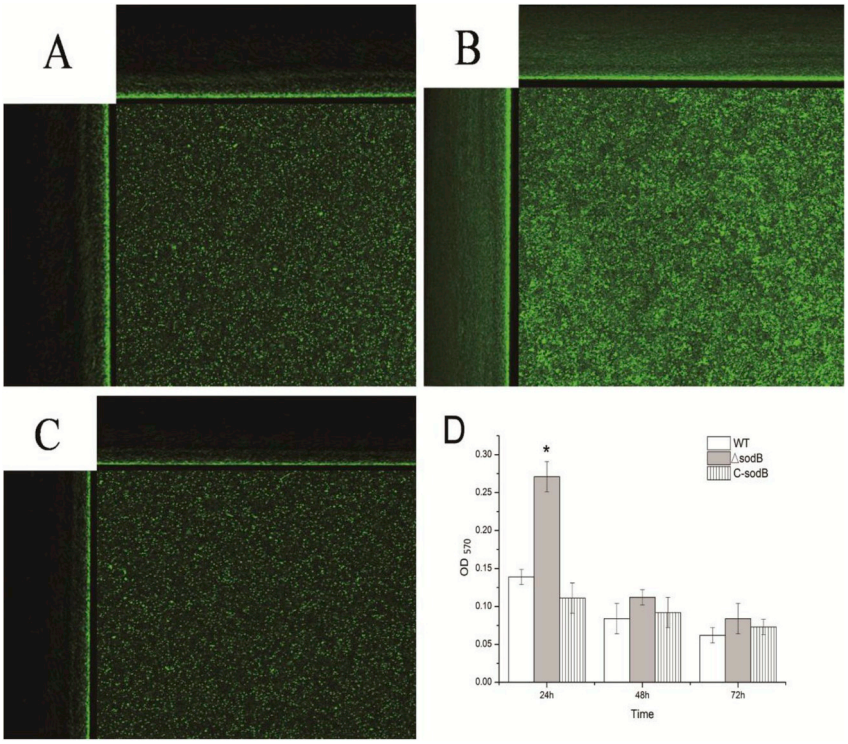

Fig. 4. The biofilm formation of *V. alginolyticus*. (A–C), WT, Δ*sodB* and C-*sodB* at 24 h by laser scanning confocal microscopy; (D), the OD_{570} values of biofilm formation were detected at 24 h, 48 h and 72 h. Values are expressed as the mean ± SE.

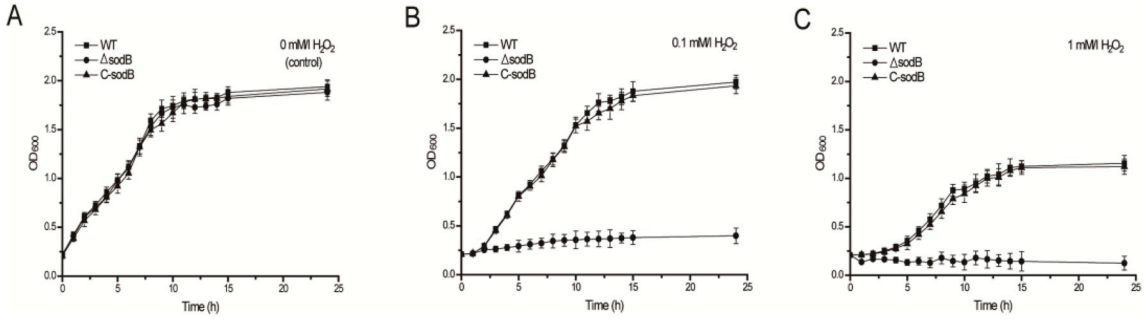

Fig. 5. Sensitivity of WT, Δ*sodB* and C−Δ*sodB* to indicated concentrations of H_2O_2 in growth. Values are expressed as the mean ± SE.

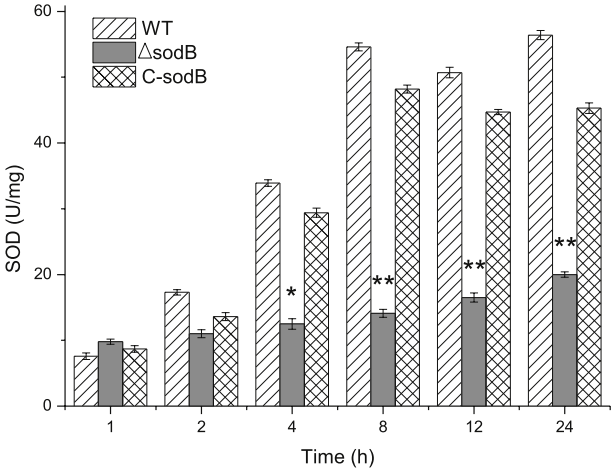

Fig. 6. SOD activities of WT, Δ*sodB* and C-*sodB* at different time-points. Values are expressed as the mean ± SE. Asterisk above bars indicate significant differences between different groups. **, $p < 0.01$; *, $p < 0.05$.

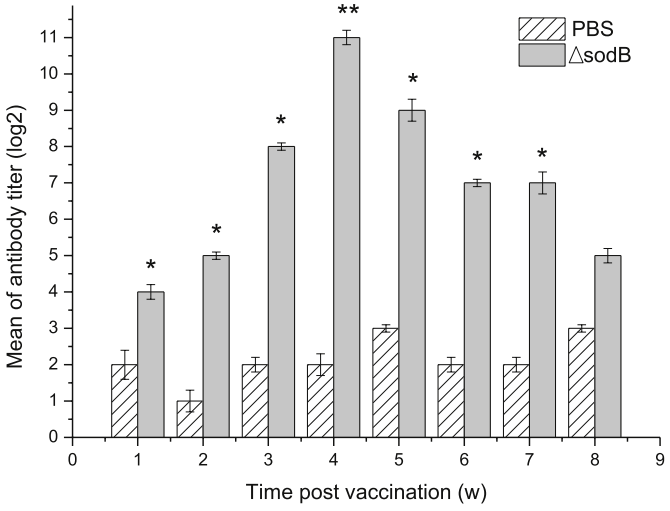

Fig. 7. Analysis of antibody levels of grouper immunized with PBS and Δ*sodB* by ELISA. Sera samples were collected at week 1–8 post-vaccination. The asterisks indicated significant differences (*$p < 0.05$, **$p < 0.01$) between the control and the immunized group.

3.5. Immunoprotection of ΔsodB in pearl gentian grouper

The immune protective efficacy of candidate live vaccine strain Δ*sodB* was evaluated in the experiment. Grouper were challenged with WT at 56 d post-vaccination with PBS and Δ*sodB*. The results showed that the fish in the Δ*sodB* group begun to die in 4th day and 87.5% fish survived until the end of the experiment with a RPS of 86.5%

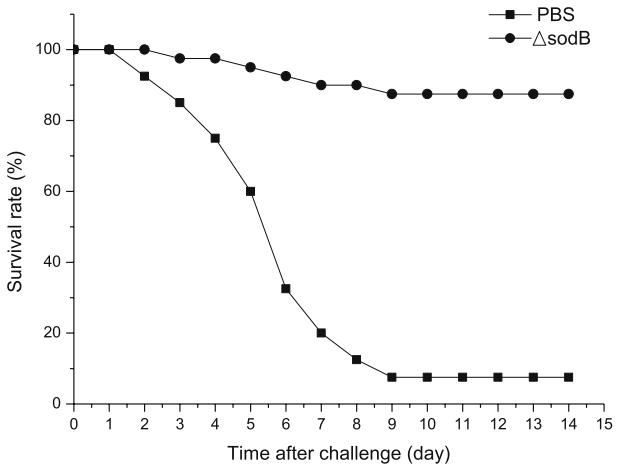

Fig. 8. survival curves of vaccinated fish after challenge with WT.

($p < 0.01$). Nevertheless, the fish in PBS group begun to die in 2nd day and only 7.5% fish survived (Fig. 8).

3.6. Histological analysis of liver and spleen post-immunization

Histopathological sections were prepared to prove the safety of strain Δ*sodB*. There was no mortality during the whole immunization experiment. Compared with PBS group, fish immunized Δ*sodB* did not exhibit obvious symptoms of hemorrhaging, septicemia, dark skin, and ulcers on the skin surface, and sample of Δ*sodB* group showed no histologically abnormality. However, distinct histological changes were observed in the samples of WT group. In liver: hemorrhage, enlargement, the nucleus enlarged and the cytoplasmic ratio reduced obviously; In spleen: hemorrhage, enlargement, inflammation and splenic cord were unclear, the red pulp area increased(Fig. 9).

4. Discussion

SOD is a kind of metal enzyme, widely spread in organisms. It can catalyze the disproportionation of superoxide anions, specifically eliminate excess superoxide anions and balance oxygen free radicals. The activity of SOD is considered to be an important indicator to evaluate oxidation resistance [25–27]. In the present study, we found SOD activity in Δ*sodB* significantlydecreased during 4–24 h during the incubation compared with that of WT and C-*sodB*. The SOD activity in Δ*sodB* decreased probably due to the deleted gene in Δ*sodB*, which affects the normal structure and function of SOD.

Hassett et al. [14] found *sodB* markedly affected aerobic growth of *Pseudomonas aeruginosa*, and the *sodB*-deletion mutant grew more slowly than wild-type bacteria. However, in this study, the growth curve of Δ*sodB* had no difference compared with that of WT. Further study showed Δ*sodB* was more sensitive to the presence of H_2O_2, which

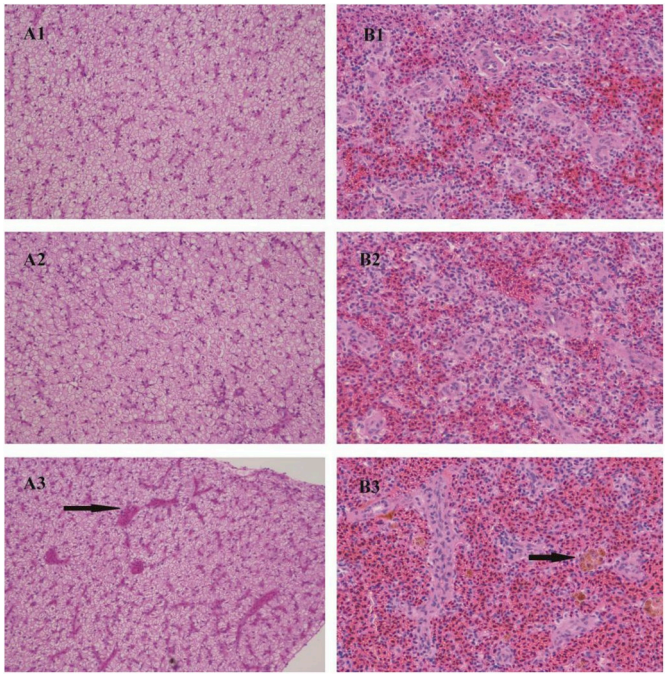

Fig. 9. Histological changes of liver (A) (200 × magnification) and spleen (B) (400 × magnification) tissues stained with Haematoxylin - Eosin. (A1, B1), PBS group; (A2, B2), Δ *sodB* group and (A3, B3), WT group.

suggested *sodB*-deletion could weaken the resistance of *V. alginolyticus* to the free radicals in the external environment. The similar results also were observed in the study of Bakshi et al. [15].

Biofilm formation is an ancient and universal characteristic of communities of microorganisms - a structural survival strategy that microorganisms cooperate to form. Biofilm is also an essential means for microorganisms to adapt to the complicated and changeable environment and resist environmental adverse factors [28–30]. Some studies showed the increasing of reactive oxygen species (ROS) could promote biofilm formation in *Klebsiella pneumoniae* [31]. In the present study, we found SOD activity was suppressed and biofilm formation was increased in *V. alginolyticus* because of *sodB*-deletion. We speculated the suppression of SOD activity increased intracellular oxidative stress in Δ*sodB*, and the increasing of intracellular oxidative stress further strengthen biofilm formation because Δ*sodB* may need to re-establish the primary defense line against adverse environmental factors by promoting the formation of biofilm due to the suppressed SOD activity.

Extracellular protease (ECP), including protease, hemolysin and siderophore, is closely related to the pathogenicity of *V. alginolyticus* [32,33]. In this study, ECP activity of Δ*sodB* significantly increased compared with WT and C-Δ*sodB*. The data suggest Δ*sodB* may be a positive contributor to activity of ECP in *V. alginolyticus*. The adhesion of bacteria to the surface of host cells is a necessary condition for pathogenic bacteria to infect the body, which is in connection with subsequent colonization, invasion, reproduction and production of toxin [34]. The swarming mobility dominated by flagellum mediates the bacteria to overcome the surface tension to reach and adhere to the surface of objects, so as to facilitate infection and biofilm formation [35]. In this study, Δ*sodB* showed lower adherence to CIK cells and suppressed swarming mobility, and these results could contribute to the attenuated toxicity of Δ*sodB* in pearl gentian grouper.

Live attenuated vaccine have been showed a great potential in preventing bacteria diseases for its outstanding advantages - weaken pathogenicity and sustained protection [16–19,36,37]. Bacterial SODs are considered to be important virulence factors, which protect bacteria against macrophages and polymorphonuclear leukocytes in the host [38,39], and thus served as an candidate for further vaccine development. In the present study, the RPS of pearl gentian grouper vaccinated with Δ*sodB* was 86.5%, and strong antibody response could be induced by Δ*sodB* without inducing clinical symptoms and obvious pathological changes, which indicated Δ*sodB* could activate long-lasting B cell-mediated humoral immune response and exhibited a significant protection for pearl gentian grouper against vibriosis.

Taken together, we have successfully constructed an in-frame deletion strain of *sodB* and the investigation showed that Δ*sodB* could increase biofilm formation, ECPase activity and sensitivity to hydrogen peroxide, and decrease swarming motility, adherence in CIK cells, SOD activity and virulence. Moreover, an effective protection against *V. alginolyticus* can be provided by Δ*sodB* vaccination. The results may benefit our understanding of the role of *sodB* in physiology and pathogenicity of *V. alginolyticus*, and offer an insight to the development of live attenuated vaccine for preventing vibriosis.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgment

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