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The role of *sodA* and *sodB* in *Aeromonas hydrophila* resisting oxidative damage to survive in fish macrophages and escape for further infection

Meimei Zhang, Yingxue Qin*, Lixing Huang, Qingpi Yan**, Leilei Mao, Xiaojin Xu, Suyun Wang, Mengmeng Zhang, Liwei Chen

Key Laboratory of Healthy Mariculture for the East China Sea, Ministry of Agriculture, Fisheries College, Jimei University, Xiamen, 361021, China

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ABSTRACT

Several bacteria have been defined as extracellular pathogens; however, in recent years, it has been confirmed that they have the ability to survive and escape the attack of host phagocytes, thus causing further infection. Previous studies have shown that *Aeromonas hydrophila* could survive in fish macrophages; however, the mechanism remains unknown. In this study, *sodA* and *sodB* of the strain *A. hydrophila* B11 were stable silenced by shRNA. The survival rates of intracellular *sodA*-RNAi and *sodB*-RNAi decreased by 91.8% and 74.9% and the immune escape rates decreased by about 32% and 92% respectively. At the same time, reactive oxygen species (ROS) in fish macrophages that phagocytosed *sodA*-RNAi and *sodB*-RNAi increased by 40% and 32.6%, respectively, compared to those of macrophages that phagocytosed the wild-type strain. Compared to *sodA,* the expression of *sodB* predominates in *A. hydrophila* without oxidative stress; however, when exposed to oxidative stress, the magnitude of up-regulation of *sodA* expression is significantly higher than that of *sodB*. With increased of methyl viologen concentration, the survival rates of *sodA*-RNAi and *sodB*-RNAi were significantly decreased. The expressions of *sodA* and *sodB* did not affect the growth of *A. hydrophila* without oxidative stress, but the inhibition of *sodA* and *sodB* expression led to a slight decrease in bacterial growth under oxidative stress. These results indicated that (1) *sodA* and *sodB* play an important role in the process of bacterial resistance to ROS damage in host phagocytic cells, allowing them to survive or even escape fish macrophages; (2) the *sodB* expression was dominant in *A. hydrophila* without oxidative stress, the *sodA* expression was up-regulated more significantly under oxidative stress, and *sodA* and *sodB* contributed equally to the process of bacterial resistance to ROS; (3) *sodA* and *sodB* complement each other and cooperate in the process of intracellular survival of bacteria to protect against ROS damage.

1. Introduction

Aeromonas hydrophila is a ubiquitous Gram-negative bacterium thats normally inhabit the aquatic environment and is commonly associated with a wide variety of diseases such as gastroenteritis, septicemia, and necrotizing fasciitis in different animals, especially in aquatic animals [1]. Previous research reports have confirmed that *A. hydrophila*-induced septicemia is one of the most severe diseases in fish farming and often leads to significant economic losses [2–4]. Many factors such as adhesins, cytotoxins, and hemolysins as well as biofilms formation, specific metabolic pathways, and virulence factor expression regulation have been reported to be associated with the virulence of *A. hydrophila* [1,5]. However, the pathogenic mechanism of *A. hydrophila* has not yet been fully clarified and a considerable amount of conflicting data exists in the literature of *A. hydrophila* virulence factors. The infections caused by *A. hydrophila* still lack effective antimicrobials.

Previous studies suggested that *A. hydrophila* can resist the killing by phagocytes, which leads chronic and recurrent infections [6]. In a previous study, this group found that the strain *A. hydrophila* B11 can survive in fish macrophages for at least 24 h, suggesting that *A.*

Abbreviations: A. hydrophila, *Aeromonas hydrophila*; Cm, chloramphenicol; DMSO, dimethyl sulphoxide; *E. coli*, *Escherichia coli*; FCS, Foetal calf serum; LB, Luria-Bertani (medium); MIC, minimum inhibitory concentration; MV, methyl viologen; NBT, nitroblue tetrazolium; OD, optical density; ORF, open reading frame; p, plasmid; PBS, phosphate-buffered saline; ^{R}, (superscript) resistance/resistant; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; ROS, reactive oxygen species; Sm, streptomycin; Tc, tetracycline; TSA, tryptic soy agar; TSB, trypticase soy broth; -, denotes connection; +, denotes complementation; △, deletion

* Corresponding author.
** Corresponding author.
E-mail addresses: yxqin@jmu.edu.cn (Y. Qin), yanqp@jmu.edu.cn (Q. Yan).

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hydrophila can use fish macrophages as shelter to resist macrophage-mediated damages and elicit further infection [7]. However, the mechanism with which *A. hydrophila* resists phagocytic killing has not yet been reported.

The production of reactive oxygen species (ROS) is a necessary effector response with which phagocytes destroy intracellular microbes [8]. Resisting the killing by ROS can help bacteria to persist inside host phagocytes where they can even grow and replicate [9]. Superoxide dismutase (SOD) constitutes a ubiquitous class of antioxidant defence metalloenzymes that catalyse the conversion of superoxide radical ions into dioxygen and hydrogen peroxide to protect cells from damage of superoxide radical ions [10]. SOD has been classified based on the type of metal bound at the active site: Cu/Zn-SOD, Mn-SOD, Fe-SOD, and Ni-SOD [11,12]. In general, Mn and Fe SODs are intracellular/cytosolic, while Cu−Zn SODs are extracellular/periplasmic [13]. It has been reported that bacteria contain one to three SOD enzymes, which can be expressed simultaneously. *Listeria monocytogenes* produces a single MnSOD, *A. hydrophila* ATCC 7966 contains both MnSOD and FeSOD, and *Escherichia coli* produces MnSOD, FeSOD, and Cu-ZnSOD [12,14–16]. MnSOD, FeSOD, and Cu-ZnSOD are known to be encoded by *sodA*, *sodB* and *sodC* respectively [15].

In the genome of *A. hydrophila* B11, two superoxide dismutase genes *sodA* (accession numbers MH379839) and *sodB* (accession numbers MH379840) have been found. However, the role of both SODs in *A. hydrophila* survival in fish macrophages and the escape from them is still remains completely unknown. In the present work, the expression of SODs was silenced to explore the functions of *sodA* and *sodB* in the *A. hydrophila* resistance to oxidative damage for the survival in fish macrophages and the escape of phagocytosis.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The sources of strains and plasmids used in this study are listed in Table 1. *A. hydrophila* strains were grown in closed tubes in tryptic soy broth (TSB) (Difco, Detroit, USA) or on tryptic soy agar (TSA) (Difco, Detroit, USA) at 28 °C, while *E. coli* strains were cultured in Luria–Bertani (LB) broth at 37 °C.The overnight bacterial culture was diluted 100-fold with fresh medium, and then shaken for 12 h on a shaker at 37 °C or 28 °C to obtain a fresh bacterial cultures. For the wild-type strain B11, the antibiotic used was 100 μg/mL streptomycin (Sm); for strains *sodA*-RNAi and *sodB*-RNAi, the antibiotics were 100 μg/mL Sm and 34 μg/ml chloromycetin (Cm).

2.2. Stable gene silencing

The stable gene-silencing assay for *A. hydrophila* was implemented based on Darsigny et al. [18] and Choi and Schweizer [19]. The first step was the acquisition of *A. hydrophila* competent cells. Simply, the overnight cultured *A. hydrophila* was inoculated into fresh LB medium (1:100 v/v) until the OD_{600} was 0.3–0.5; then, thoroughly washed twice with ice-cold sterile water at 4 °C, and subsequently washed once in ice-cold 10% washing buffer (10% redistilled glycerol, 90% sterile water, v/v); finally, the competent cells were re-suspended in 1 mL ice-cold 10% glycerol. The second step was two short hairpin RNA sequences, targeting the coding region of *sodA* and *sodB* mRNA, were designed (Table 2) and synthesized by Shanghai Generay Biotech Co., Ltd. (Shanghai, China). Then, the annealed double-stranded oligonucleotide was ligated with the pACYC184 which was digested by *Bam*HI and *Sph*I. The recombinant plasmids pACYC184-*sodA* and pACYC184-*sodB* were confirmed by sequencing and were then transformed into competent *A. hydrophila* cells via electroporation [20]. The empty plasmid pACYC184 was used as control. The stable silenced clones were screened by LB plates contained chloromycetin.

2.3. RNA extraction and reverse transcription

Ribonucleic acid (RNA) was isolated from bacteria samples using TRIzol (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instruction. The concentration and purity of RNA was evaluated by a multimode microplate reader (SYNERGYH1, Bio-Tek Instruments, INC) and its integrity was determined by agarose gel electrophoresis. 5 μg of template was used for complementary deoxyribonucleic acid (cDNA) synthesis using a RevertAid Mu-MLV cDNA Synthesis Kit (TransGen Biotech, China), according to manufacturer's instruction.

2.4. qRT-PCR

The expression of *sodA* gene and *sodB* was done by qRT-PCR using QuantStudio 6 Flex (Life Technologies, Grand Island, NY, USA). Expression levels were normalized to 16S rRNA and then calculated by the $2^{-\Delta\Delta Ct}$ method; primer sequences are listed in Table 3.

2.5. Macrophage isolation and A. hydrophila survival and escape in fish macrophages in vitro

Healthy tilapia (*Oreochromis spp*) were purchased from a commercial fish farm. Head kidney macrophages were isolated as the method described by Secombes [21] and Do et al. [22] with slight modifications. Briefly, fish were euthanized with MS-222 (Sigma-Aldrich, St. Louis, USA) and head kidney was aseptically removed. Head kidney tissue was homogenized in 10 vol of Leibovitz L-15 medium (Gibco) containing 10 IU/mL heparin, 100 IU/mL streptomycin/penicillin (S/P), and 2% fetal calf serum (FCS) and gently passed through 100 μm sterile nylon mesh. Then the cell suspension was layered on a 34/51% discontinuous Percoll (Amersham Pharmacia Biotech, UK) density gradient with a syringe and centrifuged at 400 × *g* for 30 min at 4 °C without using brakes. The macrophages were collected from the interface and washed twice with phosphate-buffered saline (PBS), then centrifuged at 400 × *g* for 10 min. The collected macrophages were seeded in a 6-well culture plate (Nunc, China) and each well containing 2 mL of L-15 medium supplemented with 10% FCS and 100 IU/mL S/P. After 4 h of incubation at 28 °C, the non-adherent cells were removed by

Table 1
Strains and plasmids used in this study.

Strain or plasmid	Genotype and/or phenotype	Source or reference
Plasmid		
pACYC184	(Cm^R Tc^R)	Provided by Prof.Nie
pACYC184-*sodA*	pACYC184 derivative containing a 60 bp fragment of one short-hairpin RNA sequence targeting the coding region of *sodA* mRNA	This study
pACYC184-*sodB*	pACYC184 derivative containing a 60 bp fragment of one short-hairpin RNA sequence targeting the coding region of *sodB* mRNA	This study
Strains		
B11	Wild-type strain (Sm^R), isolated from diseased *Anguilla japonica*	[17]
sodA–RNAi	*sodA* was silenced by shRNA (Sm^R, Cm^R)	This study
sodB–RNAi	*sodB* was silenced by shRNA (Sm^R, Cm^R)	This study
E.coli DH5α	F^-, φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA1 endA1*, *hsdR17* (*rK*$^-$, *mK*$^+$), *phoA*, *supE44*, λ$^-$, *thi -1*, *gyrA96*, *relA1*	Takara

Table 2
Oligonucleotides used to produce shRNA for stable gene silencing.

Target	shRNA sequence
sodA F	GATCCCATCACAGTCGCCATCACCATTCAAGAGATGGTGATGGCGACTGTGATGTTTTTTGCATG
sodA R	CAAAAAACATCACAGTCGCCATCACCATCTCTTGAATGGTGATGGCGACTGTGATGG
sodB F	GATCCCGCACATCTCCCAGGAAACTTTCAAGAGAAGTTTCCTGGGAGATGTGCGTTTTTTGCATG
sodB R	CAAAAAACGCACATCTCCCAGGAAACTTCTCTTGAAAGTTTCCTGGGAGATGTGCGG

Table 3
Primers for qRT-PCR.

Gene	Primers for qRT-PCR
16S rRNA F	5′TAATACCGCATACGCCCTAC3′
16S rRNA R	5′GGACCGTGTCTCAGTTCCAG3′
sodA F	5′CGCCATCACCAGACTTACATCA3′
sodA R	5′ GCCTCAAACCCGTCCAGATC3′
sodB F	5′ CTTCGCCGAGTTCAAGGAT3′
sodB R	5′ GGTTGCGGAAGTCGATGTAG3′

washing the cultures twice and the macrophages (10^7 cells/mL) were incubated in L-15 medium containing 10% FCS with the wild-type and silent strains of *A. hydrophlia* for 1 h at 28 °C [multiplicity of infection (MOI)] = 100 (100 bacteria per macrophages added). After incubation at 28 °C for 1 h, the macrophages were washed twice with cold PBS, and the cells were resuspended in 2 mL PBS. Then, the cell suspensions were treated with 250 mg/mL gentamycin for 20 min at 28 °C to eliminate extracellular bacteria, and followed by washing twice with PBS. The supernatant was withdrawn and tested for sterility. The cells were re-suspended in fresh L-15 medium with 10 IU/mL heparin, 100 IU/mL S/P, and 10% FCS, and this time point was denoted as 0 h. Then, the 0 h samples were further incubated for 1 h and 3 h and the samples were denoted as 1 h and 3 h, respectively. After that, the cells from 0 h to 3 h samples were centrifuged for 5 min at 100 × *g* at 28 °C, and 1 mL sterile distilled water was added for 30 min to lyse the cells. The CFU of the precipitate was determined by plating appropriate dilutions on TSA plates [23,24]. For the 3 h sample, the precipitate of macrophages was removed by centrifugation and the CFU of bacteria in the supernatant were counted on TSA plates. The intracellular survival rate was defined as the number of CFU at 1 h divided by the number of CFU at 0 h. The escape rate was defined as the number of CFU of supernatant at 3 h divided by the number of CFU at 0 h [24,25].

2.6. Detection of ROS in fish macrophages by nitro blue tetrazolium (NBT)

The ROS in macrophages was measured by NBT as previously described with minor modifications [26,27]. The head-kidney macrophage (1×10^6 cells per well) were collected according to the assay described above and the purified cell suspension was transferred to 96-well plates at 10^6 cells per well. The cells were incubated with 10^8 CFU of *A. hydrophila* for 2 h at 28 °C, harvested, and resuspended into the same volume of PBS. Then, a mixture of cells and bacteria was incubated with 500 μL of NBT (1 mg/mL, Sigma Chemical Company MO, USA) per well at 28 °C for 1.5 h. The supernatants were discarded, and the cells were fixed with 100% (v/v) methanol for 15 min. Each well was washed twice with 125 mL of 70% (v/v) methanol. The fixed cells were allowed to air-dried. The reduced NBT (in the form of the blue precipitate formazan) was dissolved using 120 mL of 2 N potassium hydroxide (KOH) and 140 mL of dimethyl sulphoxide (DMSO, SigmaeAldrich, St. Louis, MO, USA) per well. The turquoise-blue solution was measured with enzyme-linked immunosorbent assay, and ELISA reader at a wavelength of 630 nm.

2.7. Expressions of sodA and sodB and bacterial survival under methyl viologen (MV) stress

MIC by the standard tube dilution technique was performed in accordance with the Clinical Laboratory Standards Institute (formerly National Committee for Clinical Laboratory Standards) (CLSI 2006). All dilutions and the spots were performed in triplicate. Then all tubes were incubated at 28 °C for 24 h. The lowest concentration of MV with no bacterial growth was defined as MIC.

The expression of *sodA* and *sodB* and the bacteria survival rate under different MV concentrations were measured as previously reported [28,29]. Bacterial cultures were grown in TSB at 28 °C to the exponential phase, harvested, and resuspended into the same volume of TSB. MV was added to a final concentrations of 10 mM, 20 mM, and 30 mM (MIC is 40 mM). After 3 h of co-culture, the number of CFU was determined for each culture before and after MV treatment by plating appropriate dilutions. The RNA of the wild type strain was extracted to analyse the expressions of *sodA* and *sodB* by RT-qPCR. The percentage of survival was defined as the number of CFU after treatment divided by the number of CFU prior to treatment.

2.8. Growth curves under the MV stress

The overnight cultured the wild-type and silent strains were diluted to an appropriate concentration (OD_{600} nm = 0.03) and added to a final concentrations of 0 mM, 10 mM, 20 mM, and 30 mM MV in a 96-well plate, respectively. The control group was sterile liquid LB medium. The 96-well plate was placed in a multimode microplate reader to measure bacterial growth curves. The OD_{600} nm values of 0, 1, 2, 4, 8, 16, 24, 32, 40 and 48 h were selected for the growth curves. From the OD_{600} nm data, growth curves were plotted that compared the wild-type and mutant strains. Three independent biological replicates were performed for each data point.

2.9. Soft agar plate motility assay

Soft agar plates were prepared using LB supplemented with 0.5% agar (Difco Detroit, USA). Strain B11, *sodA*-RNAi and *sodB*-RNAi were grown in LB broth through mid-log phase. 1 μL/drop of each treatment group were then spotted in triplicate on soft agar plates with containing concentrations of 0 mM, 10 mM, 20 mM, and 30 mM MV respectively and incubated at 28 °C with periodic observation and measurement of growth circles for up to 20 h. Three technical replicates within each biological replicate were performed per group.

2.10. Data processing

All results were presented as mean ± standard error of the mean (SEM). Comparisons between the groups were performed by one-way analysis of variance (ANOVA) followed by Tukey test using SPSS software (version 20, IBM Corporation, Armonk, NY, USA). The *P*-values < 0.05 were regarded statistically significant [30,31].

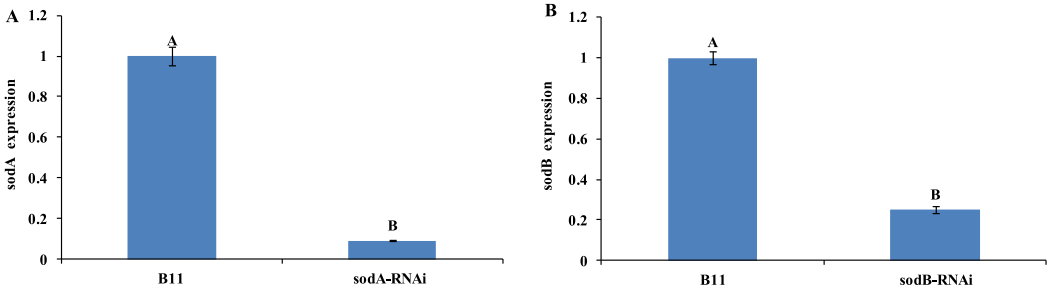

Fig. 1. Expression of *sodA* in *sodA*-RNAi and *sodB* in *sodB*-RNAi. The data are presented as the means ± SD; six independent biological replicates (three technical replicates within each biological replicate) were performed per group. The means of the treatments not sharing a common letter are significantly different at $P < 0.05$.

3. Results

3.1. Effects of stable gene silencing on the expressions of sodA and sodB

The results of shRNA-mediated gene silencing in *A. hydrophila* are showed in Fig. 1. The data indicates that the expression of *sodA* in *sodA*-RNAi and *sodB* in *sodB*-RNAi was significantly reduced by 91.8% and 74.9%, respectively, compared to the wild-type strain. These results confirmed that both *sodA* and *sodB* had been successfully silenced, indicating that *sodA*-RNAi and *sodB*-RNAi could be used for the following study.

3.2. Effects of sodA and sodB expressions on A. hydrophila survival in and escape from fish macrophages

The data in Fig. 2 showed that one hour after the bacteria were phagocytosed by fish macrophages, 38.8% of cells of the wild-type strain B11 survived, while only 14.1% cells of *sodA*-RNAi and 12% cells of *sodB*-RNAi survived. In other words, the survival rate decreased by about 64% and 69% after inhibition of the expression of *sodA* and *sodB*. All these data suggested that the expressions of *sodA* and *sodB* is directly involved in the *A. hydrophila* survival in host macrophages.

The effects of *sodA* and *sodB* expression on *A. hydrophila* escape from fish macrophages are displayed in Fig. 3. 3 h after the bacteria were phagocytosed, 31.8% cells of strain B11 could escape from fish macrophages and grow on plates, while only 21.5% cells of the *sodA*-RNAi and 2.5% cells of the *sodB*-RNAi could escape and grow. In other words, the immune escape rate decreased by about 32% and 92%, respectively, after the expression of *sodA* and *sodB* was inhibited. This indicated that *sodA* and *sodB* expression also affects the escape of *A. hydrophila* from fish macrophages, which can possibly trigger a new round of infection.

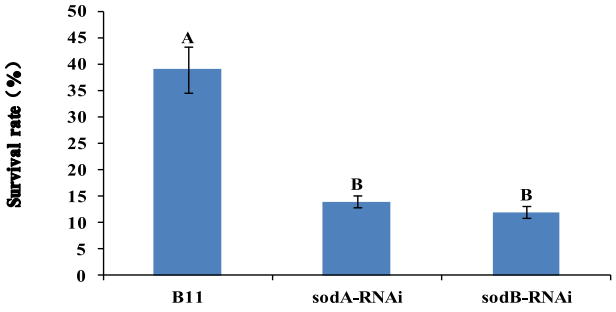

Fig. 2. Intracellular survival rate of strains B11, *sodA*-RNAi and *sodB*-RNAi. The data are presented as the means ± SD; three independent biological replicates (three technical replicates within each biological replicate) were performed per group. The means of treatments not sharing a common letter are significantly different at $P < 0.05$.

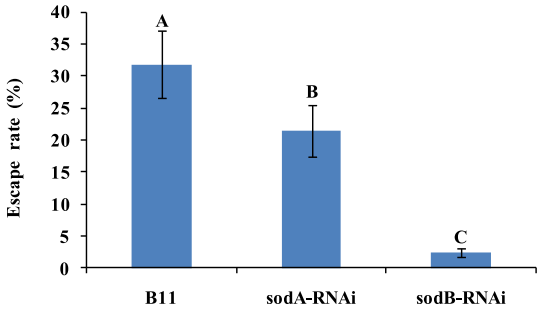

Fig. 3. Immune escape rate of strains B11, *sodA*-RNAi and *sodB*-RNAi. The data are presented as the means ± SD; three independent biological replicates (three technical replicates within each biological replicate) were performed per group. The means of treatments not sharing a common letter are significantly different at $P < 0.05$.

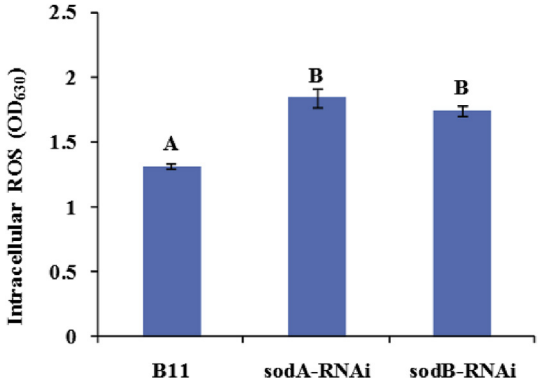

Fig. 4. ROS in fish macrophages after phagocytosing bacteria. The data are presented as the means ± SD; three independent biological replicates (three technical replicates within each biological replicate) were performed per group. The means of treatments not sharing a common letter are significantly different at $P < 0.05$.

3.3. Effects of bacterial sodA and sodB expression on the intracellular ROS level of fish macrophages

The data in Fig. 4 show that ROS in fish macrophages that phagocytosed *sodA*-RNAi increased by 40% and increased 32.6% in fish macrophages that phagocytosed *sodB*-RNAi compared to macrophages that phagocytosed wild-type strain. These results suggested that when the expression of *sodA* and *sodB* was inhibited, the ability of bacteria to resist cellular ROS decreases, which leads to increased ROS in the macrophages, and bacteria are subjected to stronger oxidative stress.

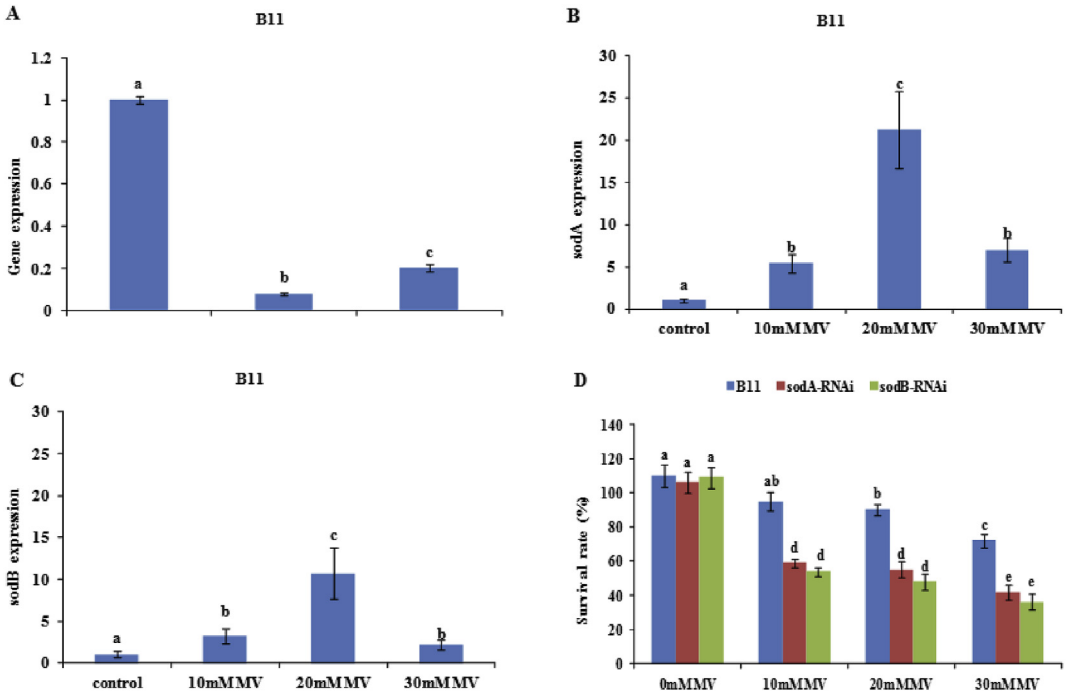

Fig. 5. Expressions of *sodA* and *sodB* under MV stress and the resulting effect on bacterial survival. (A) Expressions of *sodA and sodB* in *A. hydrophila* B11without oxidative stress. (B) Expression of *sodA* in *A. hydrophila* B11 under MV stress. (C) Expression of *sodB* in *A. hydrophila* B11 under MV stress. (D) Survival rates of different *A. hydrophila* strains under MV stress. The data are presented as the means ± SD; three independent biological replicates (three technical replicates within each biological replicate) were performed per group. The means of treatments not sharing a common letter are significantly different at $P < 0.05$.

3.4. Expressions of sodA and sodB and bacteria survival rate under MV stress

Fig. 5A shows that the expression of *sodB* is 2.5 times that of *sodA* in strain B11 without oxidative stress. From Fig. 5 B and C show that at an MV concentration of 10 mM, the expression levels of *sodA* and *sodB* increased by 4.4-fold and 2.2-fold, respectively, and the expression of both increased by 20-fold and 9.7-fold, respectively, under 20 mM MV. When the MV concentration further increased to 30 mM, these two increased by 6-fold and 1.1-fold, respectively (MIC is 40 mM). These results indicate that the expression of *sodB* predominates in *A. hydrophila* compared to *sodA* without oxidative stress. The expression of *sodA* is rapidly up-regulated under oxidative stress, and the magnitude of up-regulation was higher than *sodB*. Correspondingly, with increased MV concentration, the survival rate of the wild-type strain remained at a relatively high level, while the survival rates of *sodA*-RNAi and *sodB*-RNAi decreased significantly (Fig. 5D). No significant difference was found in the survival rates between *sodA*-RNAi and *sodB*-RNAi, indicating that both genes have equivalent effects under oxidative stress.

3.5. Effects of sodA and sodB expressions on bacteria growth

The results in Fig. 6 show that the growth curves of the three strains were coincided without oxidative stress. Under MV stress, the growth ability of the wild-type strain B11 was slightly higher than that of other strains. These data indicated that the expression of *sodA* and *sodB* did not affect the growth of *A. hydrophila* without oxidative stress, but inhibition of the expressions of *sodA* and *sodB* leads to a slight decrease in bacterial growth under the oxidative stress. This suggests that the growth ability of *sodA*-RNAi and *sodB*-RNAi in fish macrophages may be slightly affected compared to B11.

3.6. Effects of sodA and sodB expressions on bacteria motility

Previous studies suggested that motility helps bacteria to escape from host macrophages [32,33]. To exclude the impact of different motile capabilities between the three strains under the different concentrations of MV on the impact of the escape ability of bacteria from fish macrophages, bacterial motility of these three strains were measured. The results in Fig. 7 show that the difference in the motility between these three strains was not significant, regardless of presence or absence of oxidative stress. This indicates that the difference of bacteria in escape from fish macrophages was not due to the differences in their motile ability.

4. Discussion

Studies on bacterial SODs have suggested that the function of MnSOD, FeSOD and Cu/ZnSOD differs. Intracellular MnSOD and FeSOD was proposed to largely remove intracellular or metabolic sources of superoxide, while the periplasmic/extracellular Cu/ZnSOD directly combats superoxide from the animal host [10]. However, a study of the fish pathogen *Aeromonas salmonicida* detected that a periplasmic MnSOD and the authors proposed that this MnSOD may play a role in the defence against external ROS such as the periplasmic Cu/ZnSOD found in *E. coli*, *Legionella pneumophila*, *Haemophilus influenza* and *Haemophilus parainfluenzae* [34]. In *E. coli*, *sodA* was reported to be induced by many oxidative stress-related stimuli such as paraquat and H_2O_2, while *sodB* was constitutively expressed [12]. In *A. hydrophila* ATCC 7966, *sodB* was also reported to be constitutively expressed, but MnSOD was only detected during the stationary growth phase under high aeration. When induced by a lack of iron, paraquat had no detectable effect on *sodA* expression [16]. In this study, in the absence of oxidative stress, the expression of *sodB* was found to predominate. Under oxidative stress stimuli, the expression of *sodA* was up-regulated stronger, which is similar to the results reported for *E. coli* but differs from the *sodA* expression of *A. hydrophlia* ATCC 7966. This suggests that FeSOD of *A. hydrophlia* B11 may be dedicated to eliminating normal metabolic superoxide, while MnSOD could be responsible for the removal of excessive superoxide under oxidative stress.

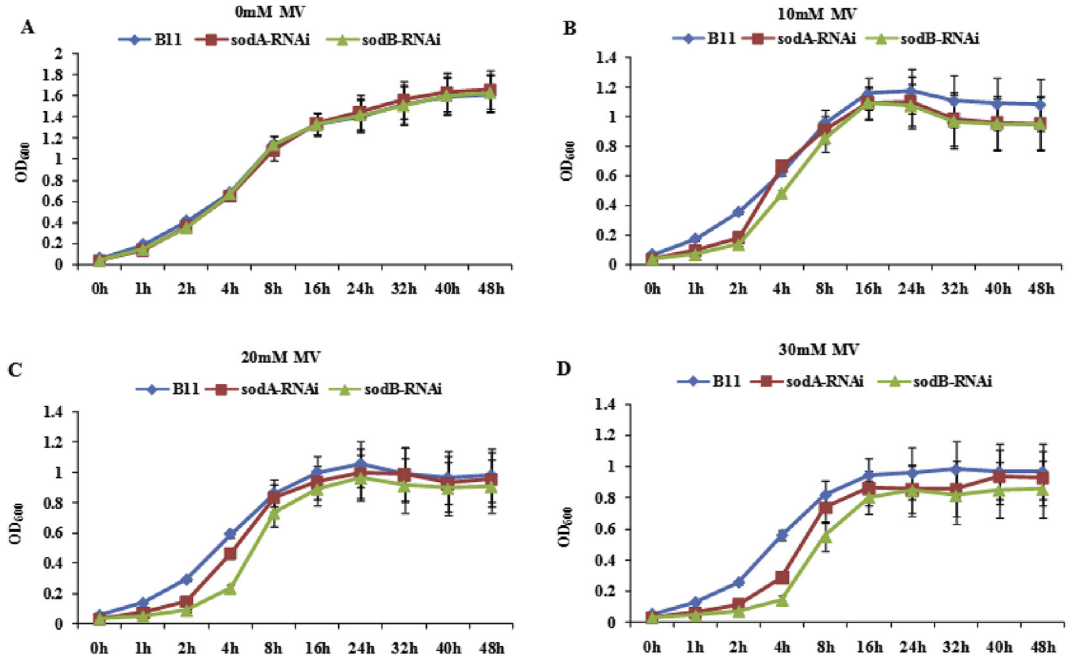

Fig. 6. Growth curves of *A. hydrophila* B11, *sodA*-RNAi and *sodB*-RNAi. The data are presented as the means ± SD; three independent biological replicates (three technical replicates within each biological replicate) were performed per group.

However, reports on the functional comparison of *sodA* and *sodB* in bacteria are still rare. Leclère et al. [16] suggested that MnSOD of *A. hydrophlia* ATCC 7966 play a role in the defence against external ROS like the periplasmic Cu/ZnSOD found in *E. coli*. The results of the present study show that there was no significant difference in the survival rate between *sodA*-RNAi and *sodB*-RNAi under the different MV concentration stress. This suggests that *sodA* and *sodB* have a similar contribution in *A. hydrophila* B11 defence against ROS damage. It can be speculated that by this way, bacteria can avoid to waste energy for excessively SOD expression under normal conditions and quickly protect themselves against oxidative stress when they are exposed to it. For bacteria, this represents a sophisticated adaptation to oxidative stress.

Since SOD has the ability to quench superoxide radicals, the role of SOD in bacterial survival in host macrophages has been widely reported. The *sodC* mutant of *Burkholderia pseudomallei* showed markedly decreased survival in mouse macrophages, and reduced numbers of bacteria were recovered from human polymorpholear neutrophils when compared to the wild-type [35]. The FeSOD mutant strain of *Edwardsiella tarda* was found to be more sensitive to H_2O_2-induced oxidative damage and less resistant against serum-and macrophage-mediated bacterial killing [36]. The survival of the *sodA* deletion mutant of *Streptococcus suis* type 2 in RAW264.7 macrophages was only half of that of the wild-type strain [37]. All these reports indicated that SOD is critical for the survival of pathogens in host phagocytic cells. The results of the present study also show that when the expression of *sodA* and *sodB* is inhibited, the intracellular survival rates of *A. hydrophila* decreased greatly. At the same time, ROS in macrophages that phagocytosed *sodA*-RNAi and *sodB*-RNAi increased significantly. The expressions of *sodA* and *sodB* in strain B11 and the survival rates of strains B11, *sodA*-RNAi, and *sodB*-RNAi under MV stress further confirmed that the expression of *sodA* and *sodB* determines the survival ability of *A. hydrophila* under MV stress. In addition, under MV stress conditions, the growth ability of *A. hydrophila* also decreased when the expression of *sodA* and *sodB* was inhibited. These results indicate that the expressions of *sodA* and *sodB* help *A. hydrophila* to resist ROS stress in macrophages, which enables their survival in or even their escape from host macrophages.

Previous studies have shown that bacterial motility is associated

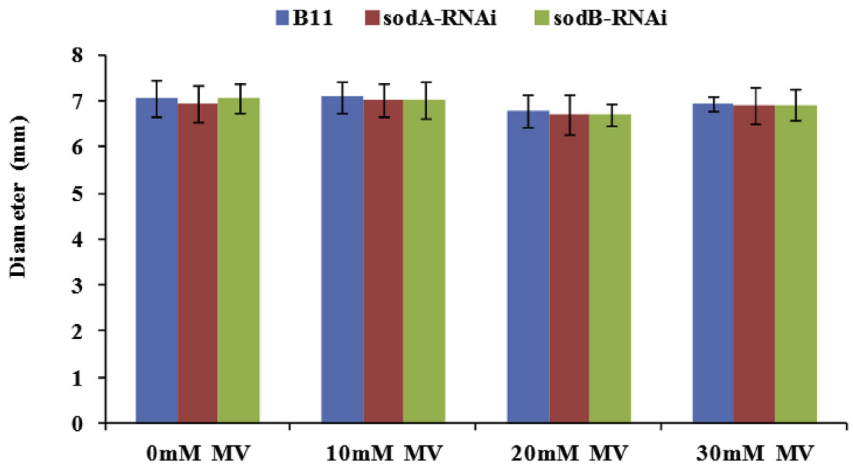

Fig. 7. Motility of *A. hydrophila* B11, *sodA*-RNAi, and *sodB*-RNAi. The data are presented as the means ± SD; three independent biological replicates (three technical replicates within each biological replicate) were performed per group.

with their ability to escape from host macrophages [32,33]. The relationship between bacterial motility and immune escape was also investigated in this study. The obtained results indicate that when the expression of *sodA* and *sodB* is inhibited, the immune escape rates of the strains *sodA*-RNAi and *sodB*-RNAi are much lower than that of the wild type strain. However, there is no significant difference was found in motile capacity between these three strains. This suggests that the difference in the ability to escape between the wild type strain and silent strains from fish macrophages is not caused by differences in their motility, but that the expression of *sodA* and *sodB* may also be involved in the escape of bacteria from macrophages. A study on *Bacillus anthracis* also suggested that catalase and SODs may play an important role for bacterial resistance oxidative damage and to escape from phagolysosomes to the cytoplasmic [38]. This suggests that when the expression of *sodA* and *sodB* is inhibited, pathogens will suffer severe oxidative damage even if they should survive the ROS oxidative stress in macrophages; consequently, their escape ability is weakened. However, the specific mechanism is still unclear and further studies are required.

5. Conclusion

The obtained results indicated that (1) *sodA* and *sodB* play an important role in the process of bacterial resistance to ROS damage in host phagocytic cells, allowing them to survive or even escape in fish macrophages; (2) the expression of *sodB* was dominant in *A. hydrophila* without oxidative stress, and the expression of *sodA* was more significantly up-regulated under oxidative stress; however, *sodA* and *sodB* contributed equally to the process of bacteria resisting ROS; (3) *sodA* and *sodB* complement and cooperate in the intracellular survival process of bacteria, which protects them against ROS damage.

Conflicts of interest

The authors declare no competing financial interests.

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