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Research Article

Small RNA sR158 Participates in Oxidation Stress Tolerance and Pathogenicity of *Edwardaiella piscicida* by Regulating TA System YefM-YoeB

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In recent years, the role of bacterial sRNAs in adversity tolerance and pathogens has attracted increasing attention. A great number of virulence-related sRNAs were reported in a variety of human pathogens. However, only a few sRNAs from aquatic pathogens were reported. In our previous study, a novel sRNA, sR158, was identified in *Edwardsiella piscicida*, an important aquatic pathogen, but its function remains unknown. In the same aquatic pathogen, we also identified a type II TA system, YefM-YoeB, in another study. In the current report, we found that the expression of *yefM-yoeB* in *E. piscicida* was regulated by sR158, which is dependent on the RNA chaperon Hfq. The deletion of sR158 reduced bacterial tolerance to oxidation pressure, enhanced bacterial capacity for biofilm formation, increased bacterial adhesion and invasion of host cells and immune tissues, and boosted bacterial general virulence, which are consistent with the effects caused by the deletion of YefM-YoeB. These findings indicate that sR158 participates in the stress resistance and virulence of *E. piscicida* by regulating YefM-YoeB. Our result is the first report that the type II TA system is regulated by sRNA, which provides new insights into the regulatory role of bacterial sRNA.

1. Introduction

Edwardsiella was isolated and identified as a new genus of *Enterobacteriaceae* in 1965 [1]. It has five species, including *E. piscicida, E. ictaluri, E. anguillarum, E. hoshinae,* and *E. tarda* [2, 3]. *E. piscicida* is an important pathogen in cultivating fisheries [4]. It is of great significance to carry out epidemiological studies on *E. piscicida* [5]. *E. piscicida* can cause infection and death in large numbers of fish [6–8]. At present, in the field of aquatic animal diseases, studies about *E. piscicida* pathogenesis are becoming more attractive.

To survive in stressful and challenging environments, bacteria have evolved sophisticated mechanisms to sense their environment and alter gene expression patterns by regulators [9]. Among various regulators, bacterial small RNAs (sRNAs) have become a research hotspot. Some sRNAs play critical regulatory roles in response to various environmental stresses, biofilm formation, pathogenicity, and other major processes [10–14].

Bacterial sRNAs are typically untranslated transcripts, 50 to 500 nucleotides in length. Regulation of target mRNAs by sRNA is achieved through base matching [15]. According to the regulation mode, bacterial sRNAs are divided into trans-coded and cis-coded sRNAs. Trans-encoded sRNA is expressed at different sites from its target genes and partially complements its target genes by a specific seed sequence. The expression site of cis-coded is the same as its unique target site, and they are completely complementary [16, 17]. Trans-encoded sRNAs often exhibit their roles in the presence of Hfq, an RNA chaperone protein [9, 18]. Our previous research has shown that Hfq in *E. piscicida* plays an indispensable role in response to stress and infection [19].

The toxin-antitoxin (TA) system is widespread in the genomes of prokaryotes and archaea but was originally discovered as a plasmid-stabilizing molecule [20, 21]. The TA operon encodes a stable toxin and an antitoxin that are easily degraded. The toxin is usually a protein, and the antitoxin can be either a protein or RNA. Up to now, seven different types of TA systems have been identified [22, 23], among which type II TA systems are a research hotspot [22]. YefM-YoeB is a common type II TA system and is involved in stress resistance in many pathogenic bacteria [24, 25]. In *E. piscicida*, YefM-YoeB is crucial to responding to adverse circumstances and pathogenicity [26].

The type II TA system is often regulated by itself [22], but almost no other regulators have been identified. In our previous study, an Hfq-dependent sRNA, sR158, was identified [27]. sR158 is located at the downstream of the TA system YefM-YoeB. In this study, the roles of sR158 in stress adaptation and pathogenicity were identified. Our study is the first to report that type II TA system expression is regulated by sRNA.

2. Materials and Methods

2.1. Strains and Growth Conditions. *Escherichia coli* S17-1λpir and DH5α grow at 37°C. *E. piscicida* TX01 (polymyxin B-resistant) grows at 28°C [26]. *E. piscicida* TX01 is cultured in an LB agar plate or in Luria-Bertani (LB), with or without certain concentration of antibiotics (polymyxin B, 100 μg/mL; chloramphenicol, 30 μg/mL).

2.2. Construction of Missing Mutant Strain. The primers are shown in Table 1. The mutant was constructed, as previously reported [26]. To obtain the mutant, ΔsR158, we constructed the deletion of 83 bp fragment of sR158 by using overlapping PCR. After amplifying two PCR fragments using primer pairs sR158KOF1/sR158KOR1 and sR158KOF2/sR158KOR2, the overlapping PCR fragments were obtained with primer sR158KOF1/sR158KOR2 and cloned into suicide plasmid pDM4, resulting in recombinant plasmid pDMsR158. The transformants were obtained by converting pDMsR158 to S17-1λpir. *E. piscicida* were conjugated with transformants. Transconjugants were selected and cultured on LB agar plates containing polymyxin B, chloramphenicol, and 12% sucrose for 48–72 h. PCR was used for screening the sucrose-resistant and chloramphenicol-sensitive colonies with primers sR158KOF3/sR158KOR3. To confirm the in-frame deletion, DNA sequencing was performed on the PCR products obtained.

Table 1: Oligonucleotide primers used in this study.

Primer name	Sequence (5′–3′)
sR158KOF1	**GGATCC**CTCTTAAGGATGCGAATC (BamH I)
sR158KOR1	TTAACCGTCATTTATCCCCGTAGTGA
sR158KOF2	AATAAATGTTAACCGTGCTGCGC
sR158KOR2	**GGATCC**ATTATTGATGTGGATGTGG (BamH I)
sR158KOF3	GCACCGCCTCGTTTACA
sR158KOR3	CGCTGGCAAAGATCAG
yefM-yoeB RTF	GATACCATGAACCGTGTAACCAACA
yefM-yoeB RTR	TAGTGAAAGCGGCAGGCAAC
hfq RTF	GAACGTGTCCCAGTTTCGAT
hfq RTR	GTGATGGTAGCTGCTGCCT

Bold letters indicate the restriction site of BamH I.

2.3. Resistance to Oxidative Stress. TX01 and ΔsR158 were grown to exponential growth phase, and then bacteria were collected and washed in PBS. About 10^5 bacteria were added per 250 μL hydrogen peroxide (3.2 mM) or PBS (control). After 60 minutes of incubation, mixture was diluted and coated with 50 μL on LB plates. These plates were incubated at 28°C for 36 h, and the number of colonies on it was then recorded. Survival rates were calculated, as described previously [26].

2.4. Biofilm and Motility Assay. The biofilm formation and motility assay were performed, as described previously [26].

2.5. Invading Host Cell Lines. FG cells and *E. piscicida* in 96-well plates were incubated for 1 h and 2 h at 25°C at a MOI of 50 : 1. After washing, the FG cells were lysed, and bacteria attached with and invaded into hose cells were examined by plate counting. Bacteria were grown in DMEM containing murine monocyte-macrophage cells, as described previously [26].

2.6. Pathogenicity Analysis In Vivo. The challenge experiment was performed, as described previously [28]. Briefly, healthy tilapias (5 groups, 40 per group) were acclimated for 2 weeks, and then were intramuscularly infected with the same dose (1×10^6 CFU) of *E. piscicida* (TX01 and ΔsR158) and PBS (control). Before collecting tissues, these fish were euthanized with 200 mg/L tricaine methanesulfonate (MS-222) (Sigma, United States). At 24 and 48 hours post-infection (hpi), five fish were dissected aseptically, and spleen and head kidney were taken for the examination of viable bacteria. The rest of the fish were observed and the number of deaths was recorded.

2.7. Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR) Analysis. RT-qPCR analysis was performed, as described previously [29].

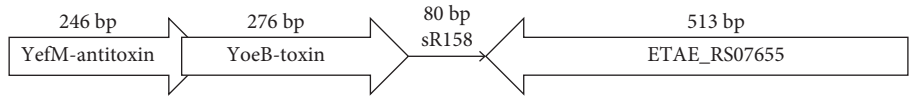

Figure 1: Genetic organization of sR158 in *Edwardsiella piscicida*.

2.8. Statistical Analysis. The experimental data were analyzed with analysis of variance (ANOVA) using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). A statistically significant difference was $P < 0.05$. All experiments were repeated three times.

3. Results and Discussion

3.1. Construction of sR158 Mutant Strain. In our previous study, 148 sRNAs of *E. piscicida* were found and identified [27]. One of them, sR158, a novel sRNA, was found to be located at the downstream of YefM-YoeB (Figure 1), which was important to oxidation pressure, biofilm formation, and virulence in *E. piscicida* [26].

To examine the function of sR158, sR158 mutant and ΔsR158 was structured by markerless in-frame. Next, we examined the effects of the sR158 deletion on the expression of *yefM-yoeB*, stress adaptation, and pathogenicity of the bacteria.

3.2. Effect of sR158 on Expression of yefM-yoeB. Since sR158 is located immediately downstream of *yefM-yoeB* and sRNA belongs to an important regulator [27], we hope to know the effect of sR158 on the expression of *yefM-yoeB*. The results of RT-qPCR showed that the expression of *yefM-yoeB* in ΔsR158 was significantly lower than that of TX01, but the expression of *ETAE_RS07655* was not affected (Figure 2), which indicates the expression of *yefM-yoeB* was upregulated by sR158. Since sR158 is an Hfq-associated sRNA and Hfq is an important RNA chaperone protein [19], we want to enquire whether this regulation of sR158 to *yefM-yoeB* expression depends on Hfq. The results of RT-qPCR showed that the expression of *yefM-yoeB* in Δ*hfq* was equivalent to that of *yefM-yoeB* in ΔsR158. These results confirm that sR158 regulates the expression of *yefM-yoeB*, whose regulatory function is Hfq-dependent. Type II TA expression is transcriptionally autoregulated by itself. For example, YefM and YefM-YoeB regulated the expression of the *yefM-yoeB* operon [26], and HigA and HigBA regulated the expression of the *higBA* operon [28]. However, as far as we know, there are no reports of sRNA regulating TA systems.

3.3. Effect of sR158 on Oxidation Stress Tolerance. Oxidative stress is an unavoidable environmental threat during the infection of the host by *E. piscicida*, and Δ*yefM-yoeB* damages the capability of bacteria to tolerate oxidation pressure [26]. Growth analysis was performed to examine the effect of sR158 on the antioxidant stress of bacteria. The growth of ΔsR158 was similar to that of wild strain TX01, indicating that the absence of sR158 did not affect its growth in normal LB medium and agar plates (Figures 3(a) and 3(b)). When cultured in LB agar plates containing H_2O_2,

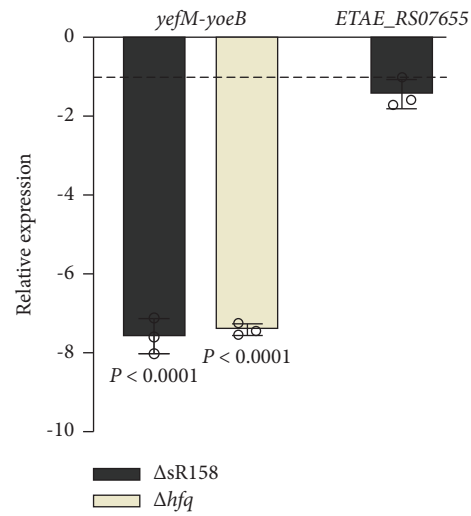

Figure 2: sR158 regulates the expression of *yefM-yoeB*. Total RNA of each strain was extracted and cDNA was synthesized. Then, the expression of *yefM-yoeB* and *ETAE_RS07655* in three strains was determined by RT-qPCR. Data are expressed as mean ± SEM ($N = 3$), where N represents the number of experiments performed.

ΔsR158 displayed obviously delayed growth compared to that of TX01 (Figure 3(c)). Consistently, the survival rate of ΔsR158 under oxidative pressure was only 32.5%, which is significantly lower than that of TX01 (65.1%) (Figure 3(d)). These results illustrate that the deletion of sR158 reduces the antioxidant capacity of *E. piscicida*. sRNA is widely involved in bacterial stress resistance [9]. For example, sRNA MicF in *E. coli* was closely associated with oxidative stress [30]. sRNA RsaC in *Staphylococcus aureus* modulates the oxidation pressure response during manganese deficiency [31]. The deletion of sRNA EsR240 reduced *E. tarda*′s survival under oxidative stress [32]. Consistently, our results showed that sR158 deficiency weakened *E. piscicida*′s tolerance to oxidation stress, probably by regulating the TA system YefM-YoeB.

3.4. Effects of sR158 on Bacterial Motility and Biofilm. As regulators of gene expression, many bacterial sRNAs participate in some important physiologies, such as motility and biofilm formation [33]. In the study of bacterial bioformation ability, it was found that the biofilm formation ability of ΔsR158 was significantly higher than that of TX01 (Figure 4(a)), indicating deletion of sR158 enhances the biofilm-forming capacity of bacteria in the community. To explore the effect of sR158 on bacterial motility after 24 h, TX01 (31 ± 1.7 mm) and ΔsR158 (32 ± 1.7 mm) showed similar movement zone diameters (Figure 4(b)), which suggests sR158 is irrelevant to bacterial motility.

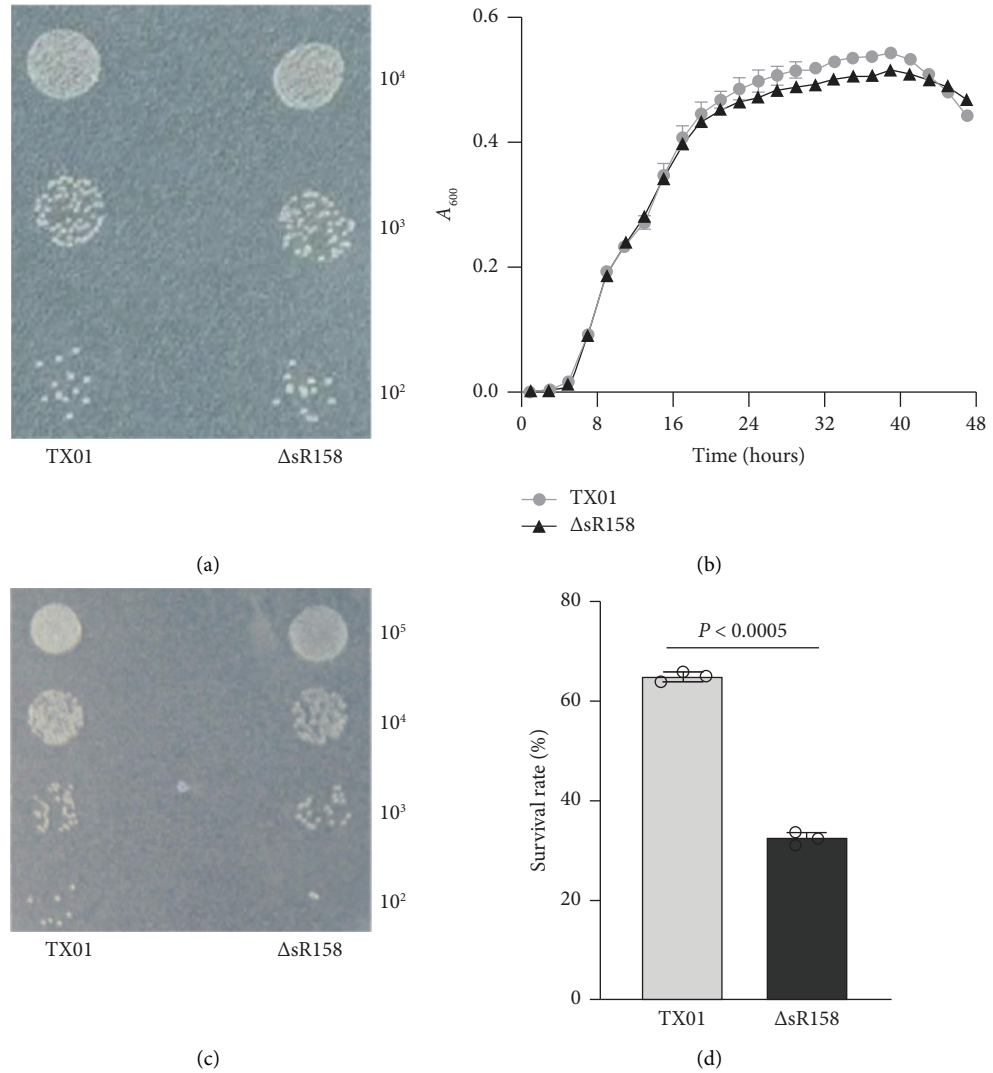

Figure 3: Resistance to oxidation stress. TX01 and ΔsR158 grew to the logarithmic phase were diluted at different concentrations, then dripped onto the normal LB solid plate (a) or LB solid plate containing 250 μM hydrogen peroxide (c), and incubated for 36 h. (b) Bacterial growth curves for TX01 and ΔsR158. (d) Logarithmic TX01 and ΔsR158 were challenged with hydrogen peroxide or PBS. Next, living bacteria were determined by plate counting. Data are expressed as mean ± SEM ($N = 3$), where N represents the number of experiments performed.

Δ*yefM-yoeB* did not affect the motility of *E. piscicida* (data not shown), which is consistent with the result of sR158. However, Δ*yefM-yoeB* enhanced bacterial biofilm formation [26], which is also in accordance with the result of sR158. This finding indicates that sR158 regulates *E. piscicida*'s biofilm formation by promoting *yefM-yoeB* expression. It has been reported that sRNA RsmZ and RsmY of *Pseudomonas aeruginosa* also regulate biofilm [34, 35]. In *Staphylococcus epidermidis*, the role of RsaE/RoxS in biofilm matrix production was also reported [36]. These reports, along with our results, manifest that sRNAs participate in bacterial biofilm formation.

3.5. Effects of sR158 on Cell Invasion and Intracellular Survival. To detect the involvement of sR158 in pathogenicity to host cells, Japanese flounder gill cells were cultured and incubated with TX01 or ΔsR158 for 2 h to detect adhesion and invasion of host cells. The results showed that the recovery amount of ΔsR158 was obviously higher than that of TX01 at 1 hpi and 2 hpi (Figure 5(a)), which indicates sR158 deficiency enhances the infection of *E. piscicida* in host cells. No significant difference was observed between ΔsR158 survival in RAW264.7 cells and TX01 at four detection time points (Figure 5(b)), suggesting sR158 is not associated with *E. piscicida* survival within host phagocytes. Consistently, the Δ*yefM-yoeB* recovered from FG cells was significantly higher than TX01. The amount of Δ*yefM-yoeB* in RAW264.7 cells was equivalent to that of TX01 [26], which further indicates the relevance of sR158 and YefM-YoeB.

3.6. Effect on General Virulence of Bacteria in Fish. In vitro experiments have shown that sR158 was involved in *E. piscicida* invasion of host cells. We hope to elucidate the function of sR158 in host infection. The results showed that

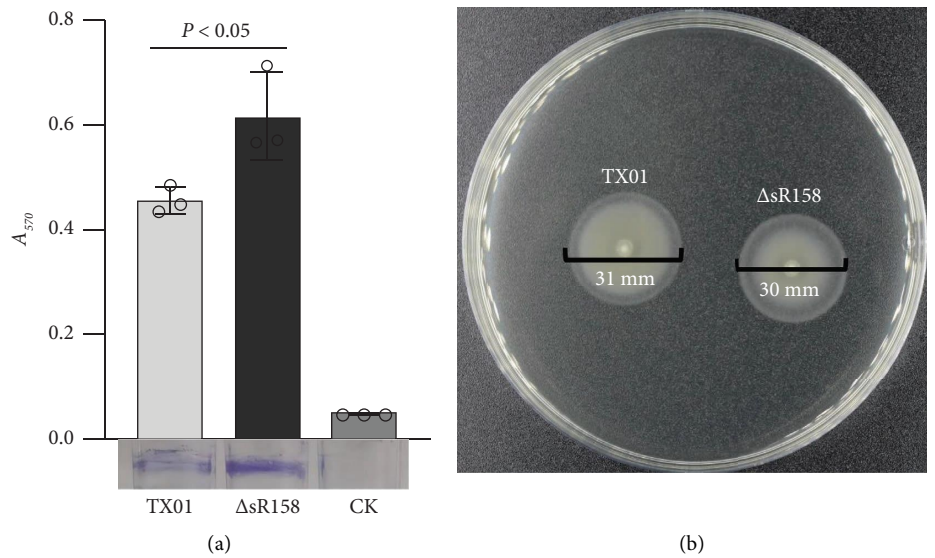

FIGURE 4: Biofilm and motility assay. (a) Biofilm assay. The two strains identified biofilm formation by crystal violet staining and measured A_{570} in the final eluent. (b) The motility of *Edwardsiella piscicida*. 1.5 μL of bacteria was dripped on the fresh swimming plate (0.3% agar). The plate was incubated to observe the bacterial movement. Data are expressed as mean ± SEM ($N = 3$), where N represents the number of experiments performed.

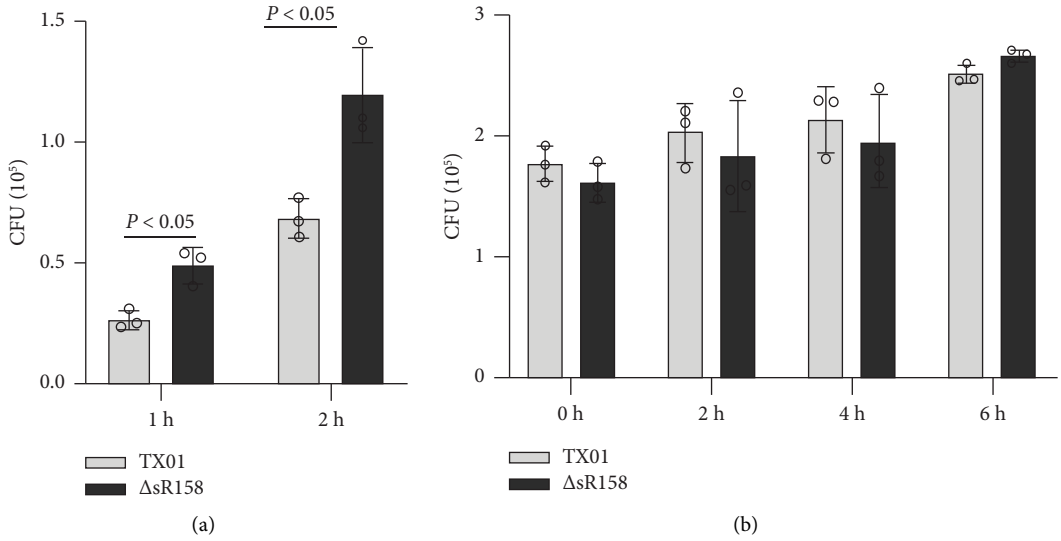

FIGURE 5: Invading host cell lines. (a) FG cells and *E. piscicida* were incubated for 1 h and 2 h. Plate counting was used to measure the number of bacteria that adhered to the cell surface and invaded the cell interior. (b) Bacteria were cultured in DMEM containing murine monocyte-macrophage cells. Lysate was added at different time points. The continuous dilution lysate was plated on an LB plate. Then, the number of bacterial colonies on the plates was recorded. Data are expressed as mean ± SEM ($N = 3$), where N represents the number of experiments performed.

the number of bacteria in ΔsR158 were significantly higher than that in TX01 (Figure 6(a)). Tilapias were infected with TX01 and ΔsR158, and the mortality of fish was monitored. The results showed that all ΔsR158-infected fish died at 16 days, while TX01-infected fish still had a 20% survival rate at 20 days (Figure 6(b)). The above results show that deleting sR158 increases the pathogenicity of *E. piscicida*.

Many bacterial sRNAs have been found to play a role in virulence in recent years. For example, in *Streptococcus pneumoniae*, multiple sRNAs were involved in niche-specific roles in virulence [37]. In *Staphylococcus aureus*, *sarA* transcript-derived sRNA teg49 regulated virulence genes independent of SarA [37]. In *Salmonella typhimurium*, sRNA IsrJ promoted bacterial invasion and enhanced the translocation efficiency of the T3SS-1 effector protein SptP into eukaryotic cells [38]. sRNA STnc150 down-regulated the protein expression of FimA, and deletion of STnc150 enhanced the bacterial adhesion ability of *S. typhimurium* to host cells and reduced LD50 in mice [39]. A great number of virulence-related sRNAs were

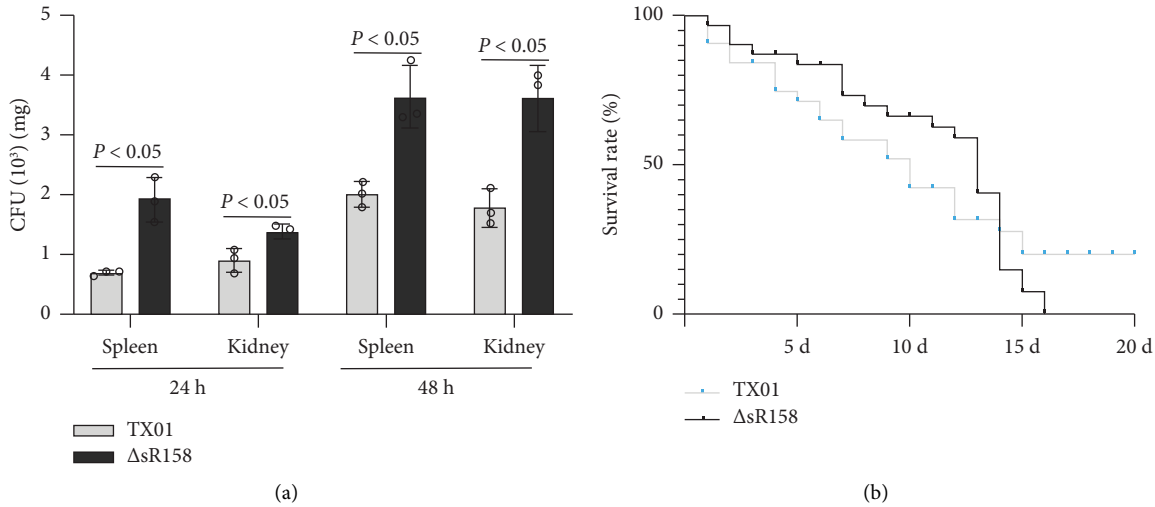

FIGURE 6: Pathogenicity analysis in vivo. (a) Experimental challenge for bacterial dissemination in vivo. Healthy fish were intramuscularly infected with the same dose of *E. piscicida* and PBS (control). After 24 and 48 hours, the spleen and kidney of fish were collected, and the amount of bacteria recovered was measured. Data are expressed as mean ± SEM ($N = 3$), where N represents the number of experiments performed. (b) For the rest of the infected tilapia (30 in each group), a 20-day mortality experiment was carried out.

reported in a variety of human pathogens, such as *Vibrio cholerae* and *V. vulnificus* [40]. However, few sRNAs from aquatic pathogens were reported. Lately, in *E. piscicida*, several sRNAs have been speculated to be involved in virulence [41]. EsR240 was confirmed to participate in *E. piscicida*'s virulence [32]. In our previous study, five Hfq-dependent sRNAs (sR012, sR043, sR082, sR084, and sR145) were involved in *E. piscicida*'s pathogenicity. The deletion of sR012, sR043, and sR082 abated bacterial virulence, but the deletion of sR084 and sR145 boosted bacterial pathogenicity [27]. In this study, the deletion of Hfq-dependent sR158 also increased *E. piscicida*'s pathogenicity, including adhesion to host cells, tissue colonization, and general virulence, which were consistent with phenotypes of the TA system YefM-YoeB deletion [26]. However, within 14 days after infection, ΔsR158 exhibited less virulence than the wild strain. We speculate the mutation of sR158 perhaps leads *E. piscicida* to be trapped in host cells and unable to spread swiftly to the whole body, causing the survival of ΔsR158 to be higher than that of the wild strain. After 14 days, since the number of ΔsR158 in tissues increased, the survival of ΔsR158 was lower than that of the wild strain, indicating the virulence of ΔsR158 was stronger than that of the wild strain.

In conclusion, our results confirm that the novel sRNA sR158 of *E. piscicida* positively regulates YefM-YoeB expression, which is Hfq-dependent. The phenotypes of sR158 deficiency are consistent with those of *yefM-yoeB*, including reduced resistance against oxidation stress, enhanced biofilm formation, increased invasion of host cells and tissues, and boosted general virulence. These findings indicate that sR158 participates in the stress resistance and pathogenicity of *E. piscicida*, probably by regulating YefM-YoeB. Our result is the first report that the type II TA system is regulated by sRNA, which provides new insights into the regulatory role of bacterial sRNA [42, 43].

Data Availability

No data were used for the research described in the article.

Ethical Approval

The study was approved by the Ethics Committee of Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences. Efforts were taken to ensure that all research animals received good care and humane treatment.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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