

Article

Construction of Green Fluorescent Protein-Tagged *Aeromonas veronii* for Understanding Its Pathogenicity in Rice-Field Eel (*Monopterus albus*)

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Abstract

The rice-field eel (*Monopterus albus*) is an important economic aquaculture species in China. *Aeromonas veronii* is one of the most common pathogens, which has a high infection and mortality rate in rice-field eel culture. In this study, a strain of *A. veronii* (AV-MA), isolated from the intestine of a diseased rice-field eel, was labelled with green fluorescent protein (GFP) using mini-Tn7-based systems. The growth curve and virulence of the GFP-labelled AV-MA (AV-MA^{GFP}) strain showed no significant differences compared to AV-MA. After immersion infection with AV-MA^{GFP} at 2.5×10^9 CFU/mL, bacterial loads in the liver, stomach, intestine, spleen, kidney, and heart tissues peaked within 1–6 h, and then declined gradually over time. AV-MA^{GFP} infection remarkably increased intestinal malondialdehyde (MDA) content, superoxide dismutase (SOD) and catalase (CAT) activities. Levels of interleukin-8 (IL-8), interleukin-10 (IL-10), Tumor necrosis factor- α (TNF- α) and nuclear factor kappa-B repressing factor (*nkrf*) mRNAs fluctuated in the intestine after infection. In conclusion, this study demonstrated that GFP DNA was inserted into the chromosome of *A. veronii* using mini-Tn7 system for tracking its invasion pathway in vivo of rice-field eels, which provides a useful tool for further studying the pathogen–host interaction mechanisms between *A. veronii* and *M. albus*.

Keywords: *Monopterus albus*; *Aeromonas veronii*; GFP; invasion pathway; intestinal immunity

Key Contribution: The mini-Tn7-mediated GFP-labelled *A. veronii* strain (AV-MA^{GFP}) exhibited growth kinetics and pathogenicity comparable to the wild-type parent strain, with stable inheritance and constitutive expression of the GFP gene. Following immersion exposure, AV-MA^{GFP} rapidly invaded multiple tissues of rice-field eels, concurrently eliciting a marked increase in intestinal antioxidant indices.



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1. Introduction

Rice-field eel (*Monopterus albus*) is one of the most important specialty freshwater fishes in China, and is widely distributed across several Asian countries [1]. It is highly prized by consumers for its delicate flavour, high-protein and low-fat content. According

to the “China Fisheries Statistical Yearbook 2025”, the total national production of rice-field eel reached 347,800 tons in 2024 [2]. However, as the high-density and large-scale culture of rice-field eel has expanded, disease control and prevention have become a major bottleneck facing the high-quality and sustainable development of rice-field eel aquaculture. Among the reported diseases including viral, fungal, bacterial, and parasitic diseases, bacterial infections represent a major challenge [3–12], although viral diseases can also have a significant impact on production [13].

Aeromonas veronii, a facultative anaerobic Gram-negative bacterium, is widely distributed in freshwater environments and has been as one of the main pathogens of farmed freshwater fishes [14]. *A. veronii* infection causes epidemic ulcerative syndrome and hemorrhagic septicemia in Nile tilapia (*Oreochromis niloticus*) [15,16], and leads to septicemia in catfishes [17,18], Roho labeo (*Labeo rohita*) [19], and Beluga sturgeon (*Huso huso*) [20]. Moreover, as an aquatic zoonotic pathogen, *A. veronii* can also infect humans and mice, and result in serious diseases such as diarrhea, gastroenteritis, and meningitis [21–24]. In recent years, an increasing number of studies have reported that *A. veronii* causes epidemic ulcerative syndrome, hemorrhagic septicemia, and mortalities in rice-field eels [3,25,26], indicating its emergence as a significant pathogen in this species. The mechanisms by which *A. veronii* invades rice-field eel and how the eel responds are still poorly understood.

Understanding the spatial distribution and colonization of pathogenic bacteria in the host is a prerequisite for elucidating pathogenesis [27]. Visualization is an effective method of studying bacterial localization in fish [28], which are conventionally carried out with microscopy, isolation culture, and radioactive tracing techniques [29]. These traditional methods have limitations for studying how pathogens invade fish: the process of visualization is cumbersome; and the signal of labelling bacteria with radioactive isotopes can be attenuated or lost with the label being metabolized or diffusing in the cell. In recent years, green fluorescent protein (GFP), an endogenous fluorescent tag, has been introduced into bacteria, which makes the bacterium visible for effectively observing the localization and tracking the pathway of invasion in fish and cultured fish cells [30–33]. Labelling bacteria is mainly conducted by transformation with a plasmid-encoding green fluorescent protein in *Edwardsiella tarda* [31], *Vibrio anguillarum* [34], *Yersinia ruckeri* [35], *A. hydrophila* [32,36], *V. splendidus* [33], and *A. salmonicida* [37]. In addition, the mini-Tn7 transposon system is a convenient tool for integration of single-copy genes into the chromosome of bacteria with single attTn7 sites [38,39]. However, this method, GFP-tagged bacteria with the mini-Tn7 transposon system, has been rarely employed to investigate the invasion pathway of pathogenic bacteria in fish.

In this study, a strain of *A. veronii* isolated from moribund rice-field eels was tagged with GFP using the mini-Tn7 transposon system, and the properties of the GFP-labelled *A. veronii* (AV-MA^{GFP}) were evaluated. With advantages of the endogenous fluorescent tag, localizations and invasion pathways of *A. veronii* were investigated in rice-field eel. Antioxidant capacity and expression of immune-related genes in intestine were analyzed for characterizing the host response to infection. This study not only demonstrated the role of GFP in tracking *A. veronii* in rice-field eel, but also provided insights into the immune response of rice-field eel after infection with *A. veronii*.

2. Materials and Methods

2.1. Bacteria and Its Culture Medium

A strain of *A. veronii* strain (AV-MA) used in this study was isolated from the visceral tissues (liver, spleen, and kidney) of diseased rice-field eels and preserved in our laboratory in May 2024. The diseased eels exhibited swollen and reddened anus, superficial hemorrhage and ulcers on the body surface, and blood exudation from the mouth and gills upon

mild compression, which were collected from a commercial farm in Suixian County, Hubei Province, China. TSB culture medium used for bacterial cultivation was purchased from Qingdao Haibo Biotechnology Co., Ltd. (Qingdao, China).

2.2. Construction of the GFP-Tagged *A. veronii*

A plasmid pUC18R6KT-mini-Tn7T-Gm-sfGFP including GFP was constructed using the pUC18R6KT-mini-Tn7T-Km vector (NovoPro, Shanghai, China) [40]. A DNA sequence containing J23100 promoter and Superfolder GFP (sfGFP) fragment synthesized in vitro was inserted into the downstream of neomycin phosphotransferase II (*npt II*) of the pUC18R6KT-mini-Tn7T-Km vector. Meanwhile, the kanamycin resistance gene (*Km^R*) DNA sequence of the original vector was replaced with the DNA sequence of gentamicin resistance gene (*Gm^R*).

Escherichia coli S17-1/ λ pir was used as the donor strain to transfer respectively the plasmid pUC18R6KT-mini-Tn7T-Gm-sfGFP and the pTNS2 helper plasmid carrying the genes encoding components of the TnsABCD site-specific transposition pathway. AV-MA and donor strains containing the plasmid pUC18R6KT-mini-Tn7T-Gm-sfGFP or the plasmid pTNS2 were cultured to the logarithmic growth phase ($OD_{600} = 0.5$), respectively. Then the three bacteria were mixed at a ratio of 1:1:1. The bacterial pellet was harvested by centrifugation at 12,000 rpm for 1 min, and resuspended in 150 μ L 10 mM $MgSO_4$. The resuspension was transferred to a sterile filter paper on a LB plate. After the resuspended solution was absorbed, the plate including the filter paper was incubated at 37 °C overnight. Then, the bacteria were transferred from the filter paper to 5 mL sterile ddH₂O by vortex thoroughly, and were collected by centrifuging the suspension. Subsequently, the bacteria were spread on a VBMM-agar plate supplemented with gentamicin (final concentration 50 μ g/mL), which was incubated at 37 °C overnight until visible colonies appeared. The colonies were imaged using a Canon EOS 80D camera (Cannon, Tokyo, Japan) under a UV light and a fluorescence microscope (BX51-34FL, Olympus, Tokyo, Japan) with a digital camera (DP-73, Olympus, Tokyo, Japan). Additionally, colonies fluorescing bright green under UV light were further validated by PCR using primers for sfGFP (sfGFP-F: 5'-AATTCGCGCCGCTTCTAGAG-3'; sfGFP-R: 5'-GCAGTTCCTACTCTCGCATGG-3') and 16S rRNA (27F: 5'-AGAGTTTGATCTGGCTCAG-3'; 1492R: 5'-ACGGCTACCTTGTT-ACGACTT-3'), which were selected as AV-MA^{GFP}.

2.3. Stability Assay of GFP Expression in AV-MA^{GFP}

A single colony of AV-MA^{GFP} was picked and inoculated into 1 mL TSB liquid medium, then cultured at 37 °C with shaking at 200 rpm for 18 h. Subsequently, 50 μ L of bacterial culture was reinoculated to 1 mL fresh TSB medium daily and continuously subcultured for 21 days. In the period of culture, 100 μ L of bacterial culture was collected at every 7-day interval, serially diluted to 10⁻⁶, and 100 μ L of the diluted suspension was evenly spread-plated onto solid TSB medium and incubated at 37 °C for 18 h. The genetic stability of GFP was examined by counting the total number of colonies and the number of colonies with green fluorescence under a UV light with 488 nm excitation wavelength.

2.4. Compare the Growth Curve of AV-MA and AV-MA^{GFP}

Single colonies of AV-MA and AV-MA^{GFP} were each picked and inoculated into 5 mL TSB liquid medium, then cultured overnight at 37 °C with shaking at 200 rpm/min. Subsequently, 500 μ L of each culture was inoculated into 50 mL fresh TSB liquid medium and cultured at 37 °C for 48 h with three parallel experiments. The OD_{600} value of the culture was measured at 0, 2, 4, 6, 8, 12, 16, 20, 24, 32, 40, and 48 h, respectively. These values were used to draw the bacterial growth curves of the two strains.

2.5. Compare LC50 of AV-MA and AV-MA^{GFP}

To assess whether the pathogenicity of AV-MA^{GFP} differs from that of AV-MA, rice-field eels (13.66 ± 0.88 g) were obtained from a commercial in Hanchuan County, Hubei Province, China. Prior to the challenge experiment, the rice-field eels were acclimated for two weeks in a recirculating aquaculture system at the Yangtze River Fisheries Research Institute in Wuhan. During the period of acclimation and experiment, the rice-field eels were kept in plastic boxes (0.57 m length $\times$ 0.44 m width $\times$ 0.32 m height). The water temperature was maintained at 25 ± 1 °C, the dissolved oxygen level was kept at 6.5 ± 1 mg/L, and the water pH was 7.0 ± 0.5 . The rice-field eels were fed once daily at a rate of 3% of their body weight during the acclimation period.

Both bacterial strains were cultured at 37 °C with shaking at 200 rpm for 18 h, followed by centrifugation at 5500 rpm for 5 min. After discarding the supernatant, the cells were resuspended in sterile PBS, and the bacterial concentration was adjusted to 5.0×10^9 , 2.5×10^9 , 1.25×10^9 , 6.25×10^8 , or 3.13×10^8 CFU/mL. In total, 390 eels were randomly selected from the acclimated rice-field eels, and randomly divided into 13 groups, with 30 fish in each group. For each bacterial strain (AV-MA and AV-MA^{GFP}), each concentration was tested for group. Each rice-field eel was infected via intraperitoneal injection with 200 μ L of bacterial suspension. Three other groups served as control, receiving intraperitoneal injections of 200 μ L sterile PBS per fish. After challenge with the two bacterial strains, the rice-field eels were maintained under the same water quality conditions (temperature: 25 ± 1 °C; dissolved oxygen: 6.5 ± 1 mg/L; water pH: 7.0 ± 0.5), which were not fed throughout the experimental period. The experiment was repeated three times independently for statistical reliability according to the above method. Mortality of each group was recorded daily for 7 days, and the semi-lethal concentrations (LC50) of AV-MA and AV-MA^{GFP} were calculated using trimmed Spearman-Kärber method [41].

2.6. Study of Invasion Pathway of *A. veronii* and Immune Response of Rice-Field Eel

Prior to the challenge experiment via immersion, 60 rice-field eels (13.57 ± 1.54 g) were acclimated in the recirculating aquaculture system for two weeks at the Yangtze River Fisheries Research Institute in Wuhan under the same water quality as mentioned above.

A single colony of AV-MA^{GFP} was picked and inoculated into TSB liquid medium, cultured at 37 °C with shaking at 200 rpm for 18 h. The culture was centrifuged at 5500 rpm for 5 min. The bacterial pellet was washed twice with sterile PBS, and resuspended in sterile PBS. An expected bacterial concentration can be obtained by adjusting the culture to a target OD600 value. A preliminary experiment revealed that rice-field eels immersed in AV-MA at a concentration of 2.5×10^9 CFU/mL could survive for more than 3 days. Therefore, 60 healthy rice-field eels were challenged via immersion with a concentration of 2.5×10^9 CFU/mL AV-MA^{GFP}. After 1 h of immersion, the eels were transferred to fresh water and reared at 25 ± 1 °C. A step-wise sequential trial was performed to monitor the temporal progression of AV-MA^{GFP} over time. At 0, 1, 6, 12, 18, 24, and 48 h post-infection, 4 fish were randomly selected ($n = 4$ per timepoint) for tissue sampling. Prior to tissue collection, the rice-field eels were humanely euthanized by immersion in a buffered solution of tricaine methanesulfonate (MS-222; Sigma-Aldrich, Shanghai, China) at a concentration of 1 mg/mL for approximately 10 min, until opercular movement ceased. Blood, stomach, intestines, liver, kidney, spleen, heart, brain, and muscle were collected under sterile conditions, weighed, and recorded. Each sample was homogenized in 1 mL sterile PBS, then gradient diluted to 10^{-4} . 100 μ L of each dilution (10^0 – 10^{-4}) was spread evenly onto TSB agar plates in triplicate and incubated at 37 °C for 18 h. The number of colonies with green fluorescence was counted under a UV light.

Simultaneously, intestinal samples were collected for determining antioxidant capacity and expression level of immune-related genes. Intestine tissues were homogenized in 0.9% saline at a 1:9 (*w/v*) ratio, and centrifuged at 2500 rpm for 10 min to obtain 10% intestine tissue homogenate. Total protein (TP), superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA) were determined according to the instructions of commercial kits from Nanjing Jiancheng Biotechnic Institute. RNA from the intestine was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The cDNA was synthesized from mRNA using a PrimeScript[®] RT Regent Kit with gDNA Eraser (Takara, Kusatsu, Japan). The expression levels of interleukin-8 (*IL-8*) (Forward: 5'-TACTGGTTCTGCTTACTGTCCG-3', Reverse: 5'-CAAATCTTTTGCCCATCCCT-3'), interleukin-10 (*IL-10*) (Forward: 5'-TTTGCCCTGCCAAGTTATGAG-3', Reverse: 5'-CATTTGGTGACATCGCTCTT-3'), Tumor necrosis factor- α (*TNF- α*) (Forward: 5'-TTTCAAGGAGGGCTGGTTCT-3', Reverse: 5'-CTTGACCAGCGCATCACTGT-3') [42], and nuclear factor kappa-B repressing factor (*nkrf*) (Forward: 5'-GTAGAGATGGTGAAGGTATCGCAG-3', Reverse: 5'-CACAAGCTCATATC-GTCCAC-3') [43] were determined according to the instructions of TB[®] Premix Ex Taq TM II kit (Takara, Kusatsu, Japan), and the total volume of each reaction system was 20 μ L. The relative expression of genes was standardized with β -actin (Forward: 5'-GCTCTCTTCCAGCCATCTTTCCT-3', Reverse: 5'-CAGACTCATCGTACTCCTGCTTG-3') [43]. The β -actin was used as the internal reference gene. The raw Ct values were first normalized to β -actin (Δ CT = CT_{target} – CT _{β -actin}). Then, the Δ CT values were further normalized to the mean Δ CT of all samples across all time points ($\Delta\Delta$ CT = Δ CT_{sample} – Δ CT_{all groups average}). The relative expression levels were calculated using the $2^{-\Delta\Delta$ CT} method and were presented as fold change relative to the overall mean.

Additionally, tissue was sampled from stomach, intestines, liver, kidney and spleen of four rice-field eels at 12 h after immersion challenge with AV-MA^{GFP}, respectively. These samples were fixed in 4% paraformaldehyde solution for histological observation using paraffin section [30]. After 20 h of fixation in 4% paraformaldehyde solution, the samples were transferred to 75% ethanol for 1 h, then dehydrated through a graded ethanol series (80% for 1 h, 90% for 1 h, 95% for 1 h, 100% for 1.5 h, and a second change of 100% for 1.5 h), cleared in xylene for 1.5 h, and infiltrated with paraffin at 56 °C for 1 h. The specimens were then sectioned at 4.5 μ m. Following slide drying, dewaxing was performed with xylene, and the sections were rehydrated through a graded ethanol series before fluorescence signal observation under a fluorescence microscope (BX51-34FL, Olympus).

2.7. Statistical Analysis

Statistical analysis was performed using JASP software version 0.19. Data are presented as mean $\pm$ standard error of the mean (SEM) unless otherwise stated. Normality of data distribution was assessed using the Shapiro–Wilk test, and homogeneity of variances was assessed using Levene's test. For comparisons at individual time points of the growth curves or at each bacterial concentration for mortality assays between the AV-MA and AV-MA^{GFP} strains, independent Student's *t*-tests were used when normality and homogeneity of variance were satisfied; otherwise, Mann–Whitney U tests (non-parametric independent *t*-tests) were applied. For bacterial load comparison, antioxidant indices, and gene expression analyses, the assumptions of normality and homogeneity of variance were first tested. When these assumptions were met, a one-way ANOVA followed by Tukey's post hoc test was used for multiple comparisons to determine significant differences between groups (statistical significance was set at *p*-value < 0.05). For data that did not meet the normality assumption, the Kruskal–Wallis test was applied instead.

3. Results

3.1. Construction of Recombinant *A. veronii* Containing sfGFP Tag

A recombinant strain of *A. veronii* containing sfGFP tag (AV-MA^{GFP}) was constructed using the mini-Tn7-based delivery system (Figure 1A). The AV-MA strain did not exhibit fluorescence under a UV light (Figure 1B). However, the AV-MA^{GFP} strain emitted a strong green fluorescent signal under both a fluorescence microscope and a UV light (Figure 1C,D). Additionally, the sizes and sequences of DNA fragment amplified with primers sfGFP-F/R and 27F/1492R were confirmed by gel electrophoresis and Sanger sequencing, respectively.

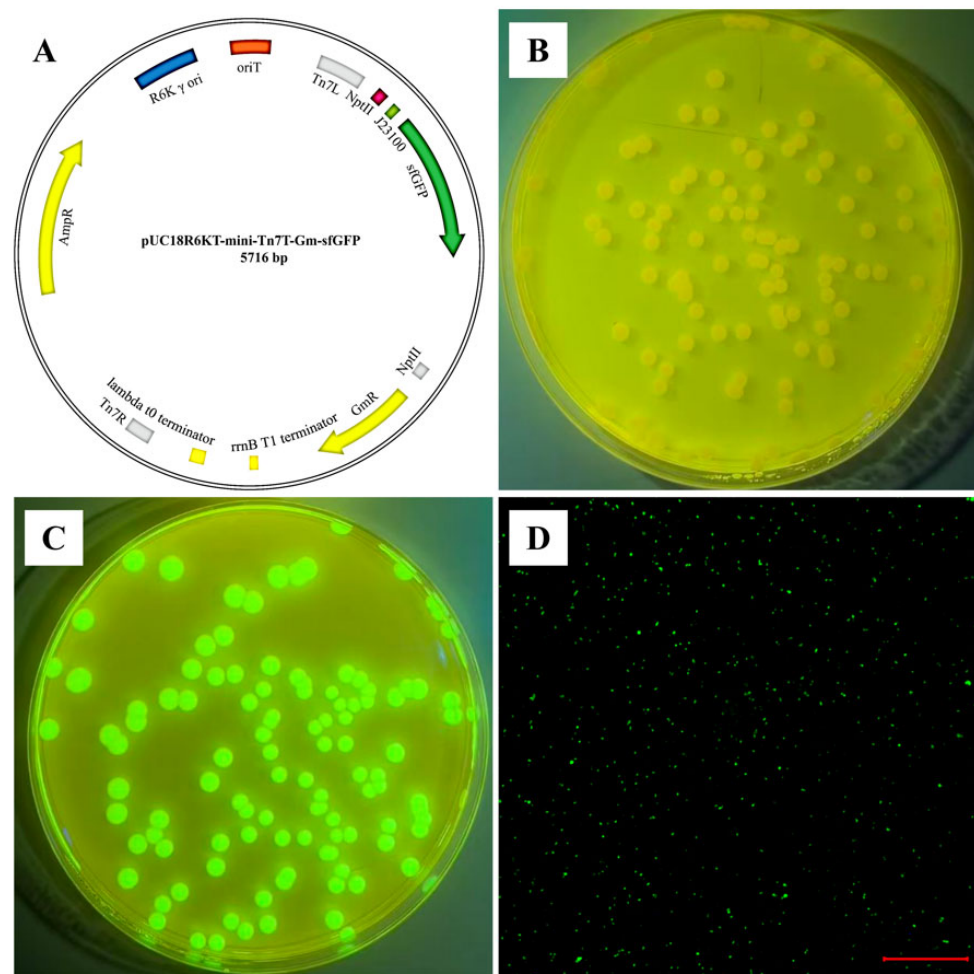


Figure 1. Recombinant plasmid and screening of AV-MA and AV-MA^{GFP} strains. (A) Map of plasmid structure; (B) the AV-MA without fluorescence under a UV light; (C) the AV-MA^{GFP} with fluorescence under a UV light; (D) the green fluorescent emission of AV-MA^{GFP} under a fluorescence microscopy. Red scale bar, 200 μ m.

3.2. Characteristics of the AV-MA^{GFP} Strain

To verify the genetic stability of GFP in the AV-MA^{GFP} strain, the strain was subcultured for 21 days. In the period of culture, the fluorescence phenotype was detected at every 7-day interval. The results showed that expression of sfGFP was stable in AV-MA^{GFP} (Figure 2). The growth curves of AV-MA and AV-MA^{GFP} strains were constructed, which showed that their curves were almost identical without significant difference (Figure 3A). The LC₅₀ of AV-MA and AV-MA^{GFP} strains were 1.81×10^9 CFU/mL and 1.52×10^9 CFU/mL, respectively, which had no significant difference. The 7-day LC₅₀ of rice-field eels respectively infected AV-MA or AV-MA^{GFP} strains were also not significantly different ($p > 0.05$) (Figure 3B). Additionally, the dead and moribund rice-field eels, infected

AV-MA or AV-MA^{GFP}, also displayed surface haemorrhage, anus red and swollen, as well as hepatic and renal haemorrhage.

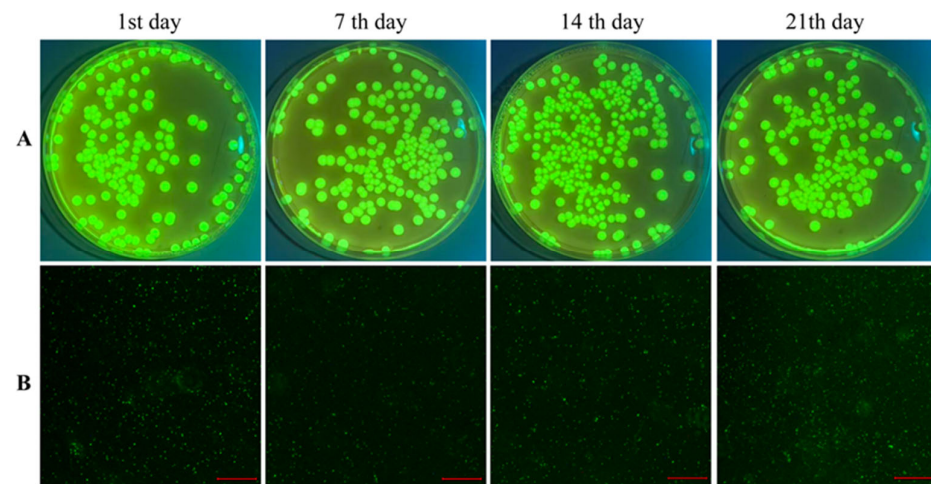


Figure 2. The fluorescence of AV-MA^{GFP} strains on different days of subculture. (A) Fluorescence of colonies on plates under a UV light. (B) Fluorescence of bacteria solution under a fluorescence microscope. Red scale bar, 200 μ m.

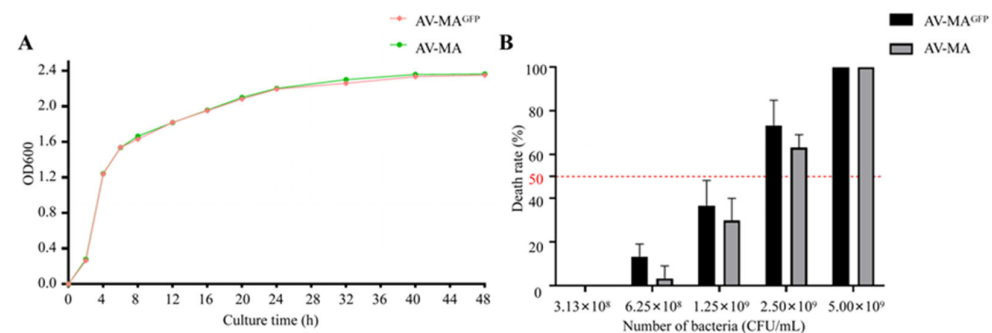


Figure 3. Assays of growth and pathogenicity between AV-MA and AV-MA^{GFP} strains. (A) Growth curve of the AV-MA and AV-MA^{GFP}. Data were presented as mean $\pm$ SEM from three independent experiments. Statistical analyses were performed using independent Student's *t*-tests; no significant differences were detected between the two strains ($p > 0.05$). (B) Death rate of AV-MA and AV-MA^{GFP}. The red line represented 50% mortality. Data were presented as mean $\pm$ SEM from three independent experiments ($n = 30$ fish per concentration per strain for each experiment). Statistical analyses were performed using independent Student's *t*-tests; no significant differences were detected at any concentration ($p > 0.05$).

3.3. Localization of AV-MA^{GFP} in Rice-Field Eel

Prior to the immersion experiment, tissue samples were taken from four randomly selected temporarily housed rice-field eels, and no bacterial colonies with green fluorescence were detected. The titers of AV-MA^{GFP} in various tissues from the rice-field eels after immersion infection was recorded by plate counting (Figure 4). The number of AV-MA^{GFP} colonies peaked at 1 h post-infection in stomach, intestine, liver, kidney, heart and muscle tissues, but 6 h post-infection in spleen. Bacterial titers gradually decreased within 24 h after infection, and were not detected in any tested tissues at 48 h post-infection. The AV-MA^{GFP} was not found in blood or brain of the rice-field eels after immersion infection. Additionally, histological observation of stomach, intestine, liver, kidney and spleen of rice-field eels was conducted at 12 h post-infection, in which robust green fluorescence was evident under the fluorescence microscope, whereas no signal was detected in the corresponding tissues of control fish (Figure 5).

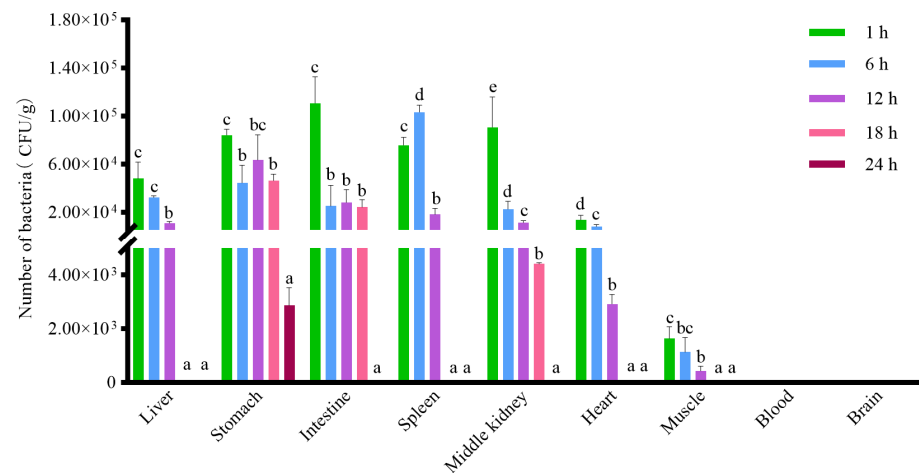


Figure 4. The titer of AV-MA^{GFP} in various tissues of rice-field eels at various times after immersion infection. Bacterial titers were expressed as CFU per gram of tissue (CFU/g). Data were presented as mean $\pm$ SEM ($n = 4$ fish per time point). One-way ANOVA with Tukey's post hoc test was performed to compare bacterial titers within each tissue individually. In each tissue, different lowercase letters above the bars denoted statistically significant differences between time points following immersion ($p < 0.05$).

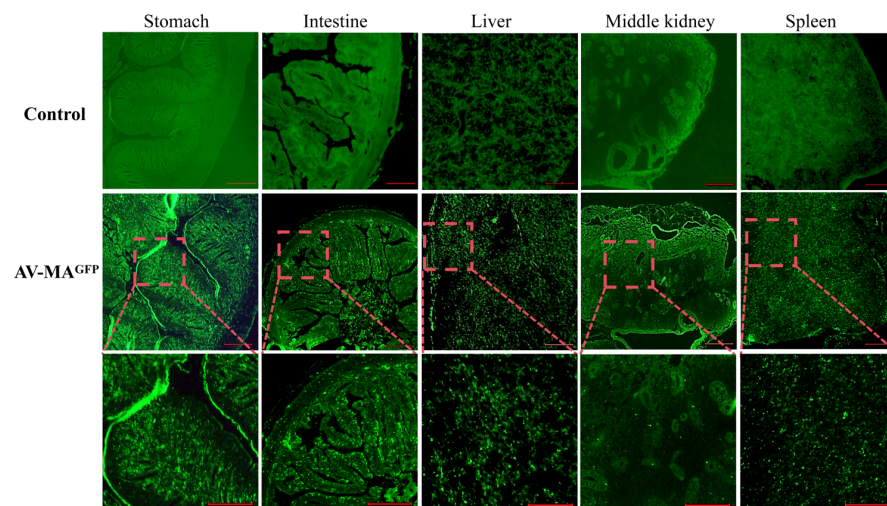


Figure 5. Tissue localization of AV-MA^{GFP} in rice-field eels at 12 h post-infection. Scale bar, 200 μ m.

3.4. Effect of Bacterial Infection on the Intestinal Immunity of Rice-Field Eel

Antioxidant indices including MDA, SOD and CAT were assayed in the intestine of the rice-field eels within 24 h after immersion infection, of which results are presented in Figure 6. Following immersion infection with AV-MA^{GFP}, intestinal MDA content, SOD activity, and CAT activity all showed significant increases compared to the 0 group, peaking at 18 h post-infection before gradually declining.

The expression levels of immune-related genes (*IL-8*, *IL-10*, *TNF- α* , and *nkrf*) in the intestine were also altered after infection (Figure 7). Notably, *IL-10* expression was significantly upregulated at 1 h post-infection, while the other tested genes exhibited varying degrees of fluctuation over the 24 h observation period. These changes collectively indicate that the host mounted an immune response following bacterial challenge.

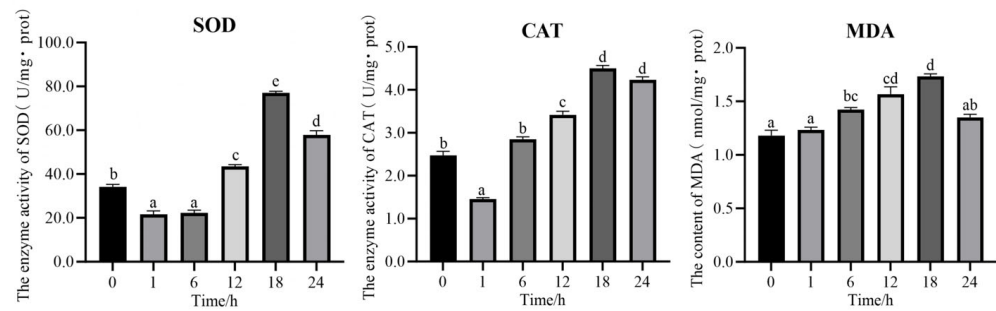


Figure 6. The influence on antioxidant indices in the intestine of rice-field eels after immersion infection with AV-MA^{GFP}. Data were presented as mean $\pm$ SEM ($n = 4$ fish per time point). Different lowercase letters above the bars indicated significant differences among time points (one-way ANOVA with Tukey's post hoc test, $p < 0.05$).

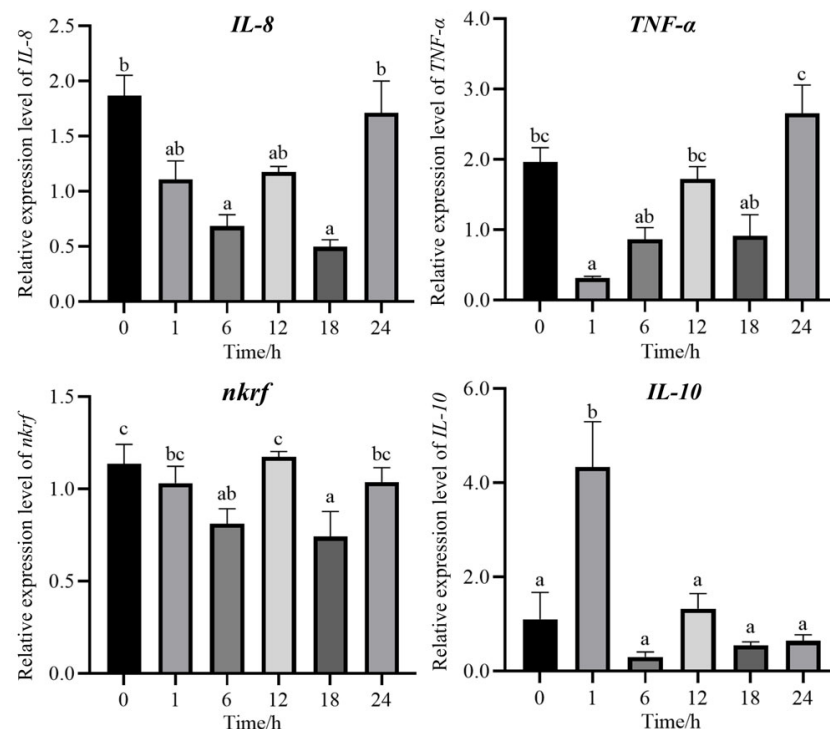


Figure 7. The expression levels of immune-related genes in the intestine of rice-field eels after immersion infection with AV-MA^{GFP}. Gene expression was first normalized to β -actin (Δ CT) and then further normalized to the mean Δ CT of all samples across all time points ($\Delta\Delta$ CT). Data were presented as mean $\pm$ SEM ($n = 4$ fish per time point). Different lowercase letters above the bars indicated significant differences among time points (one-way ANOVA with Tukey's post hoc test, $p < 0.05$).

4. Discussion

Reports have been increasing that *A. veronii* caused disease outbreaks and deaths of farmed fish across different geographic regions [44–52]. In recent years, mass mortalities in farmed rice-field eel have been documented [3,25,26]. It is necessary to strengthen studies on infection mechanism and disease control and prevention of *A. veronii* in fish with the rise of opportunistic bacterial pathogens as a global trend. Green fluorescent protein (GFP) has become a useful biomarker for visualizing bacterial invasion and dissemination in fishes [30–33]. Here, we employed the mini-Tn7 transposon system to integrate a single copy sfGFP into the chromosome of *A. veronii*. The resulting fluorescent strain was then characterized for genetic stability of the GFP, retention of virulence, and its spatiotemporal pattern of infection in rice-field eel.

GFP-tagged bacteria were mainly constructed by ligating GFP into a plasmid and its transfer into the bacteria [31–37]. However, the construct could be lost during cell division, especially without antibiotic selection, due to plasmids replicating autonomously [53,54]. The method of mini-Tn7 transposon can deliver the DNA sequence of GFP into the bacterial chromosome, which can maintain stable inheritance [39]. In this study, the strain of AV-MA^{GFP} was constructed using the mini-Tn7 system with single attTn7 sites, which could still fluorescence an intense green under a UV light when it was continuously subcultured for 21 days. Meanwhile the growth curve and LC50 were not significantly different between AV-MA and AV-MA^{GFP} strains. The dead and moribund rice-field eels infected with AV-MA or AV-MA^{GFP} exhibited clinical signs similar to the previously reported for *A. veronii* infection in this species [3]. These results showed that the AV-MA^{GFP} strain could serve as a reliable surrogate for the wild strain in subsequent investigations of pathogenesis.

Few bacteria were observed in the brain of black carp (*Mylopharyngodon piceus*) infected with an *A. hydrophila* strain transformed with a GFP-encoding plasmid [32]. A few colonies were detected in blood samples in the experiments of Crucian carp (*Carassius auratus gibelio*) [36] and black carp [32] infected with *A. hydrophila* strains with GFP. Studies of *A. hydrophila* infection in Nile tilapia suggested that *A. hydrophila* could enter into brain tissue mainly by means of phagocytes and as free colonies via blood circulation of the brain [55,56]. In our experiment of immersion infection of rice-field eel with the AV-MA^{GFP} strain, no colony was found in the plates of blood or brain within 48 h after infection; since no bacterium was detected in the blood of rice-field eels in this study, it could be that no AV-MA^{GFP} had entered into the brain tissue. The infection kinetics of AV-MA^{GFP} revealed several features relevant to its pathogenicity. The rapid dissemination to liver, kidney, heart, stomach, intestine and spleen within 1–6 h post-infection demonstrates that *A. veronii* can efficiently establish systemic infection shortly after immersion exposure. The gradual decline in bacterial loads after 6–12 h indicates effective host immune clearance within 24–48 h. In the experiment of black carp infected with *A. hydrophila* strains via immersion, high bacterial numbers were found at 4 h post-challenge, and the number of bacteria in gills and intestine was higher than other tissues within 72 h after infection [32]. Similar results were observed in the experiment of Crucian carp infected with *A. hydrophila* strains by immersion: bacteria numbers peaked at 4 h post-infection and remained high in gills and intestine within 24 h after infection [36]. In this study, apart from high bacteria numbers found in intestine and stomach, the numbers of AV-MA^{GFP} in liver, spleen and kidney were also high. These different results indicated that the infection kinetics of the AV-MA^{GFP} strain differed from *A. hydrophila*, and its infection process was rapid and quickly attacked the immune organs after infection.

The abundance of AV-MA^{GFP} in the stomach and intestine remained high at 18 h post-challenge, whereas bacterial numbers in other tissues had already declined to low or not being detected. Intestinal MDA, SOD and CAT also reached maximum levels at 18 h post-challenge. However, the MDA content was kept rising until 18 h post-challenge, whereas the SOD and CAT activities were first declined and then increased. MDA serves as a crucial indicator for assessing cellular oxidative stress levels [57]. Similar patterns of oxidative stress and antioxidant responses have been observed in other fish–pathogen systems. For instance, *Streptococcus iniae* infection in olive flounder (*Paralichthys olivaceus*) initially induced excessive reactive oxygen species (ROS) generation, which was subsequently accompanied by a transient enhancement in antioxidant enzyme activities (SOD and CAT) [58]. This parallels our observation in rice-field eels infected with *A. veronii*, where intestinal MDA levels increased significantly and SOD/CAT activities showed a transient elevation peaking at 18 h post-infection, suggesting that modulation of the antioxidant system is a common host response to bacterial infection across different fish species. The

principal role of *IL-10* seems to regulate the inflammatory response, thereby limiting host damage caused by an excessive immune response [59]. In our study, *IL-10* expression was significantly upregulated at 1 h post-challenge, and subsequently declined to the normal level. Expression of pro-inflammatory cytokines (*IL-8* and *TNF- α*) and *nkrf* seemed to be suppressed, which was decreased, returned to normal, reduced again, and then recovered once more. The results of expression of immune-related genes in intestine may indicate that rice-field eels regulated inflammation to prevent excessive inflammatory response after infection. Excessive inflammatory responses can lead to tissue damage and necrosis, which in turn may cause fish death [60]. Although we observed significant changes in intestinal antioxidant enzyme activities and immune-related gene expression following infection, our preliminary H&E staining of tissues at 12 h post-infection did not reveal overt histopathological alterations in the infected group compared to the controls (data not provided). This may be explained by the relatively mild challenge conditions used in this immersion model, as well as the possibility that biochemical and transcriptional responses precede detectable structural damage. Future investigations incorporating systematic histopathological evaluation at higher infection doses or later time points will be necessary to directly establish the correlation between bacterial colonization and tissue injury.

5. Conclusions

In this study, a strain of *A. veronii* with chromosomally inserted sfGFP (AV-MA^{GFP}) was constructed using the mini-Tn7 delivery system, for which the characteristics of growth, virulence and visible fluorescence were verified. After immersion challenge with AV-MA^{GFP}, the bacteria rapidly invaded multiple tissues of rice-field eels, especially immune-related organs including liver, spleen and kidney, and persisted longest in stomach and intestine. Analyses of intestinal antioxidant indices and immune-related gene expression confirmed that the GFP-labelled strain elicited host responses comparable to those expected for the wild-type strain. In conclusion, the AV-MA^{GFP} strain developed in this study provides a reliable visualization tool for tracking *A. veronii* colonization and dissemination in rice-field eels, laying a foundation for future investigations into the pathogenesis of this pathogen.

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Abbreviations

The following abbreviations are used in this manuscript:

GFP	Green fluorescent protein
TSB	Trypticase Soy Broth Medium
LB	Luria-Bertani Medium
VBMM	Vogel-Bonner Minimal Medium
LC50	Semi-lethal concentrations
SOD	Superoxide dismutase
CAT	Catalase
MDA	Malondialdehyde
TP	Total protein

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